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Use of cellular impedance to characterize ligand functional selectivity at G protein-coupled receptors

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Use of cellular impedance to characterize ligand functional selectivity at G protein-coupled receptors

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Résumé

Les récepteurs couplés aux protéines G (RCPGs) représentent la plus grande famille de cibles thérapeutiques pour le traitement d'une panoplie de pathologies humaines. Bien que plusieurs décennies de recherche aient permis de façonner nos connaissances sur ces protéines membranaires, notre compréhension des déterminants moléculaires de leur activité signalétique reste encore limitée. De ces domaines de recherche, une avancée récente a mis à jour un nouveau phénomène, appelé *sélectivité fonctionnelle des ligands*, qui a bouleversé les paradigmes décrivant leu fonctionnement de ces récepteurs. Ce concept émane d'observations montrant que l'activité pharmacologique de certains ligands n'est pas nécessairement conservée sur tout le répertoire signalétiques connu du récepteur et peu se restreindre à l'activation sélective d'un sous-groupe de voies de signalisation.Ce nouveau modèle pharmacologique de l'activation des RCPG ouvre de nouvelles possibilités pour la découverte de médicaments plus efficace et sûr, ciblant les RCPGs. En effet, il permet la conception de molécules modulant spécifiquement les voies signalétiques d'intérêt thérapeutique, sans engager les autres voies qui pourraient mener à des effets secondaires indésirables ou de la tolérance.

Cette thèse décrit l'utilisation d'une nouvelle approche sans marquage, basée sur la mesure du changement l'impédance cellulaire. Par la mesure des changements cellulaires, comme la morphologie, l'adhésion et/ou la redistribution des macromolécules, cette approche permet de mesurer de façon simultanée l'activité de plusieurs voies de signalisation impliqués dans ces réponses.

Utilisant le récepteur β_2-adrénergique (β_2AR) comme modèle, nous avons démontré que les variations dans l'impédance cellulaire étaient directement liées à l'activation de multiples voies de signalisation suite à la stimulation du récepteur par son ligand. L'agoniste type du β_2AR, l'isoprotérénol, s'est avéré induire une réponse d'impédance dose-dépendante constituée, dans le temps, de plusieurs caractéristiques distinctes pouvant être bloquées de façon compétitive par l'antagoniste ICI118,551 Par l'utilisation d'inhibiteurs sélectifs, nous avons été en mesure de déterminer la contribution de plusieurs voies signalétiques canoniques, comme les voies dépendantes de Gs et Gi, la production d'AMPc

et l'activation de ERK1/2, sur ces changements. De plus, la dissection de la réponse d'impédance a permis d'identifier une nouvelle voie de mobilisation du Ca^{2+} contribuant à la réponse globale des changements initiés par la stimulation du β_2AR. Dans une autre étude, nous avons rapporté que la réponse calcique induite par le β_2AR serait attribuable à une transactivation Gs-dépendant du récepteur purinergique P2Y11, lui-même couplé à la protéine Gq. La mesure d'impédance permettant de distinguer et de décrire une pléiade d'activités signalétiques, nous avons émis l'hypothèse que des ligands arborant des profils signalétiques différents généreraient des réponses d'impédance distinctes. Le criblage d'une librairie de ligands spécifiques au β_2AR a révélé une grande variété de signatures d'impédance. Grâce au développement d'une approche computationnelle innovatrice, nous avons été en mesure de regrouper ces signatures en cinq classes de composés, un regroupement qui s'est avéré hautement corrélé avec le profil signalétique des différents ligands.

Nous avons ensuite combiné le criblage de composés par impédance avec l'utilisation d'inhibiteurs sélectifs de voies signalétiques afin d'augmenter la résolution du regroupement. En évaluant l'impact d'une voie signalétique donnée sur la signature d'impédance, nous avons été en mesure de révéler une plus grande variété de textures parmi les ligands. De plus, cette méthode s'est avérée efficace pour prédire le profil signalétique d'une librairie de composés non caractérisés, ciblant le β_2AR. Ces travaux ont mené à l'élaboration d'une méthode permettant d'exprimer visuellement la sélectivité fonctionnelle de ligands et ont révélé de nouvelles classes de composés pour ce récepteur. Ces nouvelles classes de composés ont ensuite été testées sur des cardiomyocytes humains, confirmant que les composés regroupés dans différentes classes produisent des effets distincts sur la contractilité de ces cellules.

Globalement, ces travaux démontrent la pertinence de l'utilisation de l'impédance cellulaire pour une évaluation précise des différences fonctionnelles parmi les composés ciblant les RCPGs. En fournissant une représentation pluridimensionnelle de la signalisation émanant des RCPGs à l'aide d'un seul essai ne requérant pas de marquage, les signatures d'impédance représentent une stratégie simple et innovante pour l'évaluation

de la fonctionnalité sélective des ligands. Cette méthode pourrait être d'une grande utilité dans le processus de découverte de nouveaux médicaments.

Mot clés: récepteurs couplés aux protéines G (RCPGs), récepteur β_2-adrénergique (β_2AR), sélectivité fonctionnelle des ligands, réseaux signalétique, impédance cellulaire, cardiomyocytes, découverte de médicaments.

Abstract

G protein-coupled receptors (GPCRs) represent the largest family of therapeutic targets for the treatment of a wide variety of human pathologies. Decades of research have provided an extensive base of knowledge about these fascinating membrane proteins, yet significant advancements in the understanding of the structural and functional details of these important drug targets continue to accumulate to this day. One such area of research in particular that has caused a paradigm shift in the way we conceptualize receptor function is a recently identified phenomenon known as *ligand functional selectivity*. This concept refers to the numerous observations that the pharmacological activity of a ligand at a given receptor is not always conserved over all possible signalling events engaged by the receptor, often resulting in the selectivity of a ligand to modulate only a subset of the receptor's signalling repertoire. This model of receptor activity reveals exciting new possibilities for the discovery of safer and more efficacious drugs targeting GPCRs; through the design of drugs specifically targeting the pathway of therapeutic interest without modulating other, uninvolved pathways which could lead to tolerance or adverse effects.

This thesis will describe the use of a novel, label-free technique based on cellular impedance to further characterize ligand functional selectivity at GPCRs. By measuring changes in higher-order cellular responses, such as changes in morphology, adhesion and redistribution of macromolecules, this approach provides a means to simultaneously measure the activity of multiple signalling pathways converging on these responses.

Using the β_2-adrenergic receptor (β_2AR) as a model system, we have demonstrated that changes in cellular impedance reflect the activity of multiple signalling events elicited following ligand stimulation of the receptor. Isoproterenol, the prototypical agonist of the β_2AR, was found to elicit a dose-dependent impedance response consisting of multiple, discrete features over time, which could be blocked in a competitive manner by the antagonist ICI118,551. Using pathway-selective inhibitors, we were able to dissect the contribution of many of the canonical pathways activated by the β_2AR, including G_s- and G_i-dependent signalling, as well as cAMP production and ERK1/2 activation. Furthermore, through the pharmacological dissection of this impedance response, we identified a novel

Ca^{2+} mobilization pathway that contributes to the overall cellular response to β_2AR stimulation. In a separate study of the mechanism generating this β_2AR-promoted Ca^{2+} response, we revealed a G_s-dependent transactivation mechanism of the G_q-coupled P2Y11 purinergic receptor. Given the ability of impedance measurements to capture this pleiotropic signalling activity, we then reasoned that ligands exhibiting different signalling profiles should generate distinct impedance signatures. In screening a library of functionally selective compounds targeting the β_2AR, we obtained a wide variety of impedance signatures. Through the development of a novel computational approach, we were able to cluster these signatures into five distinct compounds classes, which were highly correlated with signalling profiles of the ligands.

In an extension of this approach, we then combined impedance screening with the use of pathway-selective inhibitors to determine if this would provide greater resolution in distinguishing among functionally distinct compounds. By assessing if and how a given signalling pathway contributes to a ligand's impedance signature, we were able to reveal even more texture among ligands targeting the β_2AR. Furthermore, this approach was found to be predictive of the signalling profiles of a library of uncharacterized compounds for the β_2AR. This work led to the development of a visualization method to express ligand functional selectivity and revealed potentially novel classes of compounds for the receptor. These compound classes were then validated in human cardiomyocytes, confirming that compounds clustering into different classes produced distinct effects on cardiomyocyte contractility.

Altogether, this work demonstrates the ability of cellular impedance to accurately measure functional differences among compounds targeting GPCRs. In providing a representation of the pluridimensionality of GPCR signalling using a single, label-free assay, impedance profiling represents an innovative strategy to assess ligand functional selectivity and may be a valuable addition to future drug discovery campaigns.

Key words: G protein-coupled receptor (GPCR), β_2-adrenergic receptor (β_2AR), functional selectivity, signalling networks, cellular impedance, cardiomyocytes, drug discovery

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Abbreviations

5-HT	5-hydroxytryptamine
α_2AR	α_2 adrenergic receptor
AA	arachadonic acid
AKAP	A-kinase anchoring protein
$AT_{1A}R$	angiotensin 1A receptor
β_1AR	β_1 adrenergic receptor
β_2AR	β_2 adrenergic receptor
β-arr	β-arrestin
BRET	bioluminscence resonance energy transfer
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CCK_BR	type B cholecystokinin receptor
CHO	Chinese hamster ovary
CRE	cAMP response element
CTC	cubic ternary complex
D2R	Dopamine D2 receptor
DAMGO	[D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin
DMR	dynamic mass redistribution
DVG	(Asp^1, Val^5, Gly^8)-angiotensin II
EGFR	epidermal growth factor receptor
EPAC	exchange nucleotide protein directly activated by cAMP
ERG	ergotamine
ETC	extended ternary complex

FAK	focal adhesion kinase
FRET	fluorescence resonance energy transfer
GABA	γ-aminobutyric acid
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanosine exchange factor
GGL	G protein gamma-like
GIP	GPCR interacting protein
GMP	guanosine monophosphate
GPCR	G protein-coupled receptors
GRK	GPCR kinase
GSK	glycogen synthase kinase 3
GTP	guanosine triphosphate
HDL	high density lipoproteins
HEK293	human embryonic kidney-293
HIV	human immunodeficiency virus
IGFR	insulin-like growth factor receptor
IKK	IκB kinase
Ins-1-P	inositol-1-phosphate
IP	inositol phosphate
IP_3	inositol trisphosphate
IPSC	induced pluripotent stem cell
JAK	Janus kinase
μOR	μ-opioid receptor
MAGI-2	membrane-associated guanylate kinase-like protein inverted-2

MAPK	mitogen activated protein kinase
MC4	type 4 melanocortin receptor
MD	molecular dynamics
mGluR	metabotropic glutamate receptor
MSH	melanocortin stimulating hormone
NGF	nerve growth factor
NHE3	Na^+/H^+ exchanger type 3
NHERF1/2	Na^+/H^+ exchanger regulatory factors 1 and 2
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NPA	R-(-)-10,11-dihydroxy-N-n-propylnoraporphine
PACAP	pituitary adenylyl cyclase-activating polypeptide
PAF	platelet-activating factor
PDE	phosphodiesterase
PDZ	PSD-95/Discs-large/ZO-1
PI_3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PP2A	protein phosphatase 2A
PSD-95	postsynaptic density protein 95
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide

PTH1R	type-1 parathyroid hormone receptor
RAMP	receptor activity modifying protein
RGS	regulators of G protein signalling
RhoGEF	Rho guanosine exchange factor
RKIP	Raf kinase inhibitor protein
RSK2	p90 ribosomal S6 kinase 2
RTK	receptor tyrosine kinase
SHP-1	Src homology phosphatase 1
SHP-2	Src homology phosphatase 2
SI	(Sar^1, Ile^8)-angiotensin II
SII	(Sar^1, Ile^4, Ile^8)-angiotensin II
SSNMR	solid state nuclear magnetic resonance
STAT	signal transducers and activators of transcription
TM	transmembrane domain
TPR1	tetratricopeptide repeat 1
V2R	V2 vasopressin receptor

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INTRODUCTION

1. Introduction to G protein-coupled receptors

G protein-coupled receptors (GPCRs) are an extraordinary family of cell surface receptors. With their seven transmembrane tertiary structure, they weave themselves into molecular machines perfectly tuned to transduce extracellular signals, in the form of small molecules, peptides, ions and even photons, into intracellular signalling events that allow a precise and appropriate response to a diverse environmental context. In fact, this capacity of cells to transduce extracellular signals into an intracellular response is the basis for organism multicellularity and underlies a host of fundamental and diverse cellular activities in eukaryotes. As a result, GPCRs are found in eukaryotes ranging from protists and fungi, to plants and animals (Perez, 2005).

In humans, GPCRs represent the largest family of cell surface receptors, with over 900 individual receptor subtypes (Lappano & Maggiolini, 2011). This remarkable abundance exemplifies the broad reactivity of GPCRs to an extraordinary variety of stimuli, ranging from photons and Ca^{2+} ions, to small molecules such as hormones and neurotransmitters, odorants and tastants, to peptides and even larger proteins. In accordance with such a broad spectrum of stimuli, GPCRs are involved in nearly every facet of human physiology including organism development (Katanaev, 2010), perception of sight (Palczewski, 2012), smell (DeMaria & Ngai, 2010), and taste (Palmer, 2007), modulation of mood and behaviour (Catapano & Manji, 2007), cardiac function (Salazar, Chen, & Rockman, 2007), blood vessel tone (Kauffenstein, Laher, Matrougui, Guérineau, & Henrion, 2012), innate and adaptive immunity (Yang, Chertov, & Oppenheim, 2001; Yona, Lin, & Stacey, 2010) and in the maintenance of various homeostatic mechanisms (Shioda et al., 2008; Tsunematsu & Yamanaka, 2012), among countless other essential functions.

Given their ubiquitous role in human physiology, it is not surprising that GPCRs also represent the largest family of therapeutic targets (Imming, Sinning, & Meyer, 2006; Overington, Al-Lazikani, & Hopkins, 2006; Pierce, Premont, & Lefkowitz, 2002). Whether

playing a direct role in the pathology of a given disease, or by providing an indirect mechanistic route to therapeutic benefit, GPCRs often represent an effective clinical target for the treatment of pathologies ranging from diabetes (Ahrén, 2009) to asthma (Barnes 2011), cardiovascular disorders such as hypertension (Brinks & Eckhart, 2010) and heart failure (Lymperopoulos, Rengo, & Koch, 2007), neuropsychiatric disorders like schizophrenia (Capuano, Crosby, & Lloyd, 2002) and Parkinson's disease (Xu et al. 2012), human immunodeficiency virus (HIV) infection (Maeda, Das, Nakata, & Mitsuya, 2012), pain (Pan et al., 2008) and in the treatment of various forms of cancer (Lappano & Maggiolini, 2011).

GPRCs transduce extracellular signals into intracellular effects through the modulation of an important class of cytoplasmic effector proteins – the heterotrimeric G proteins. These effectors consist of three subunits. The Gα subunit, of which there are four main families (see Table 1), act as the primary determinant of the signalling response induced upon activation of the heterotrimeric protein. The β and γ subunits, of which there are at least five and thirteen isoforms, respectively, are generally regarded as a non-dissociable complex and can elicit additional signalling events themselves (Table 1). The G protein acts as a "molecular switch" whose activity is dependent on the guanosine nucleotide bound to it. When bound by guanosine triphosphate (GTP), the heterotrimeric G protein is in its active signalling state, capable of interacting with and activating downstream effectors. When the bound GTP is hydrolyzed to guanosine diphosphate (GDP), the G protein returns to its inactive state. By possessing intrinsic GTPase activity, the G protein itself limits the duration of its signalling activity by spontaneously hydrolyzing its bound GTP to GDP. This activity cycle can be influenced by other proteins to either increase the activation of G proteins by stimulating the exchange of GDP for GTP (i.e. guanosine exchange factors, GEFs) or by stimulating the hydrolysis of GTP to GDP (i.e. GTPase activating proteins, GAPs) to return the protein to its inactive state. GPCRs exert their intracellular effect largely through the activation of G proteins. Receptor activation stimulates the exchange of GDP for GTP, thus activating the G protein to elicit downstream signalling events (Figure 1) through either the α- or βγ-subunits. GPCR subtypes possess a selectivity for distinct G proteins, which determines, to a large extent, the subsequent signalling events modulated by the

receptor and ultimately its role cellular physiology. Consequently, GPCRs were traditionally classified by the family of G proteins to which they preferentially couple; however, the notion of GPCR-G protein coupling specificity has been challenged by the observation that many (if not most) receptors can couple to and activate multiple G protein subtypes (See *Section 3.1*, page 12).

Table 1: Heterotrimeric G proteins and their signalling properties.
Adapted from Hermans (2003).

Subunit	Family	Main subtypes	Primary effector
α	α_s	$G\alpha_s$, $G\alpha_{olf}$	Adenylyl cyclase ↑
	$\alpha_{i/o}$	$G\alpha_{I1}$, $G\alpha_{i2}$, $G\alpha_{i3}$	Adenylyl cyclase ↓
		$G\alpha_{oA}$, $G\alpha_{oB}$	K^+ channels ↑
		$G\alpha_{t1}$, $G\alpha_{t2}$	Ca^{2+} channels ↑
		$G\alpha_Z$	Cyclic GMP phosphodiesterase ↑
	$\alpha_{q/11}$	$G\alpha_q$, $G\alpha_{11}$, $G\alpha_{15}/G\alpha_{16}$	Phospholipase C ↑
	α_{12}	$G\alpha_{12}$, $G\alpha_{13}$	Actin cytoskeleton
β	β_{1-5}	Different assemblies of $\beta\gamma$-dimers	Adenylyl cyclase ↑/↓
			Phospholipases ↑
			Phosphatidylinositol 2-kinase ↑
γ	γ_{1-13}		Protein kinase C ↑
			Protein kinase D ↑
			GPCR kinases ↑
			Ca^{2+}, K^+, and Na^+ channels

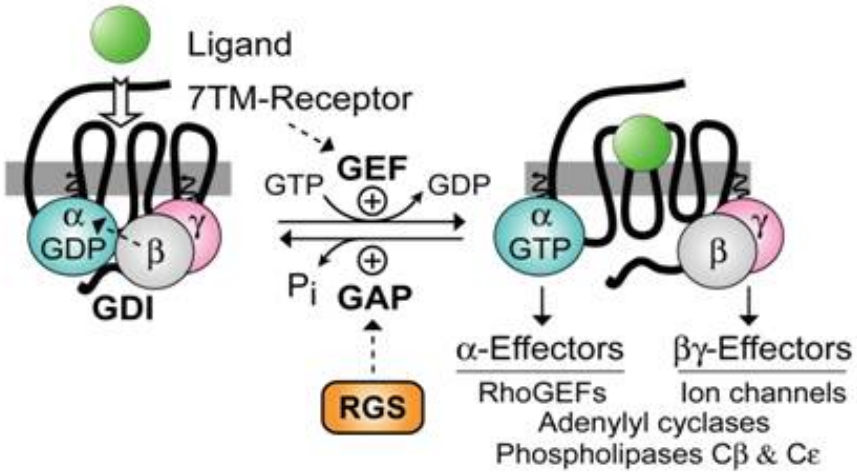


Figure 1: Mechanism of G protein activation.
Receptor activation couples to G protein activation by promoting the exchange of GDP for GTP on the α subunit of the heterotrimeric G protein. Once activated, the α- and βγ-subunits are capable of interacting independently with and activating downstream effectors to elicit a cellular response. Hydrolysis of the Gα-bound GTP to GDP, either through its intrinsic GTPase activity, or with the assistance of additional regulatory GTPase-activating proteins (GAPs, e.g. regulators of G protein signalling (RGS) proteins, see *Section 3.2.vi,* page 22), inactivates the heterotrimeric G protein, terminating its signalling activity. Adapted from Siderovski and Willard (2005)

2. The evolution of receptor theory

GPCRs have long been the cornerstone of pharmacological theory. Many of the key pharmacological concepts that are used to this day to define the properties of drugs were discovered and developed in GPCR model systems.

Much of this early work was performed by a select group of scientists working in the United Kingdom in the first half of the 20th century, including Gaddum, Clark, Schild & Stephenson, who developed the quantitative methods to measure and the theoretical approaches to

explain the response of endogenous substances or drugs (collectively referred to as *ligands*) in isolated tissues (Hill, 2006). Early measurements of concentration-response profiles in these isolated systems and the application of the law of mass action to these data (Gaddum, 1937) allowed scientists to quantitatively determine the *affinity* of a compound, or its propensity to recognize and bind to a given target (Clark, 1937). These seminal studies assumed that the tissue response to the ligand was proportional to the percentage of occupied receptors, often termed the *receptor occupancy model*. However, evidence began to accumulate indicating that such a model could not account for all of the pharmacology being observed. In 1954, E.J. Ariëns demonstrated that different agonists acting on the same receptor could produce different maximal responses in a tissue and proposed a proportionality term, the "intrinsic activity" of a ligand, to describe these quantitative differences in agonist strength (Ariëns, 1954). In 1956, R.P. Stephenson provided clear evidence of the quantitative disparity between the occupancy of a ligand and the magnitude of its pharmacological effect. Like Ariëns, he introduced a new pharmacological parameter to represent the strength of an agonist to elicit a response. He thus defined *efficacy* as the capacity of a ligand, once bound, to initiate a reaction in the receptor that culminates in a cellular response (Stephenson, 1956). To further explain this new pharmacological parameter, Stephenson claimed that:

> "Different drugs may have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses" (Stephenson, 1956)

Thus, contrary to prior assumptions, the propensity of a ligand to bind to a receptor does not solely explain its ability to generate a response through that receptor. In other words, affinity and efficacy are two independent properties of a given ligand-receptor pair. Whereas affinity provides information about the strength of the physical interaction between the ligand and receptor, which can be used to predict the selectivity of a drug for its target, it is the efficacy of a ligand that ultimately determines the magnitude and nature of the cellular response that is generated. This work ultimately helped establish a mathematical formalism to describe the response of a drug at a given receptor:

$$Response = f\left(\frac{[A] \times \varepsilon[R_t]}{[A] \times K_A}\right)$$

(Equation 1; Kenakin, 2004, Stephenson, 1956)

where ε is an empirical proportionality constant representing the intrinsic efficacy of drug A, R_t is the receptor density in the tissue, K_A is the affinity constant of the drug for the receptor. The function *f* relates the initial stimulus of the system to a tissue response.

These advancements also established the concept of *partial agonists*, or ligands that, even when occupying all of the available receptors in a system, do not elicit a maximal response as compared to a *full agonist*. The intrinsic efficacy, ε, of a ligand could vary from zero, as is true for antagonists, to a large positive value, for full agonists, or anywhere in between. The *potency* of a drug, a property that refers to concentration of the drug required to produce a response of a given magnitude, would therefore be influenced by both the affinity and the efficacy of the drug at a given receptor. The experimental and analytical techniques developed during this time were employed for decades to characterize and develop numerous drugs that remain on the shelves of pharmacies today, including the Nobel Prize winning work by Sir James Black during the 1960s for the development of beta blockers (antagonists of the β adrenergic receptors) for the treatment of angina and other cardiovascular disorders and H_2 histamine receptor antagonists to treat stomach ulcers (Black, 1988).

Despite the extraordinary power of such a model, researchers were still confounded with the biochemical nature of the efficacy parameter and its dependence on the tissue in which it is assessed for a given ligand-receptor pair. Since the precise steps linking the binding of an agonist to a tissue response could not easily be known or quantified, Black & Leff (1983) sought to derive a more practical or 'operational' approach to quantifying efficacy. With the *operational model* of pharmacological agonism these researchers provided a mathematical formalism for the relationship between the concentration of an agonist and the tissue response it generated in purely biochemical and experimentally accessible terms:

$$Response = \frac{[A] \times \tau \times E_{MAX}}{[A](\tau + 1) + K_A}$$

(Equation 2; Black & Leff, 1983)

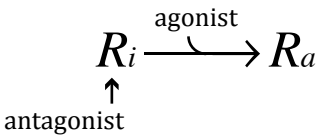
$$\tau = \frac{[R_t]}{K_E}$$

(Equation 3; Black & Leff, 1983)

Key to this new model of receptor theory is the introduction of the τ parameter, which functions as a 'transducer ratio' and is a measure of the efficiency of receptors occupied by agonist A to elicit a response in a given system. τ is inversely proportional to the concentration of occupied receptors necessary to elicit a half-maximal response in the system, K_E (Equation 3). Therefore, in systems where receptor activation is very efficiently coupled to a tissue response (i.e. relatively few receptors need to be activated to produce a response), τ would be large. Only in systems with a very high receptor number and stimulated with a strong agonist would the tissue response approach the system maximum, E_{MAX}. This mathematical formalism was extremely powerful for analyzing pharmacological systems in several ways. First, the definition of the τ parameter provided a quantitative description of tissue-specific differences in the coupling of receptor stimulation to a tissue response. Although the value of τ for a given agonist can vary from tissue to tissue, the rank order of τ among a collection of agonists should not change. Therefore, τ also provided a practical means of ranking agonists according to their *relative efficacies* regardless of the system used for the measurement.

While this conceptual framework remains very effective to explain many aspects of GPCR pharmacology to this day, receptor theory has continued to develop and provide even greater insights into mechanistic basis of drug activity at GPCRs. The operational model was developed, for example, on the assumption of a *two-state model* of receptor activation (see Equation 4). According to this "lock-and-key" type model, receptors existed in two distinct states or *conformations*: an inactive (R_i) and an active receptor conformation (R_a).

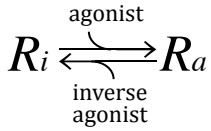
Unliganded receptors would remain in an inactive state, R_i, until bound by agonist, which would induce a conformational change to convert the receptor into an active signalling state R_a to elicit a cellular response. If bound by an antagonist, however, the receptor would remain locked in an inactive conformation, R_i, and thus not produce a cellular response, but would also prevent an endogenous agonist access to activate the receptor.



(Equation 4)

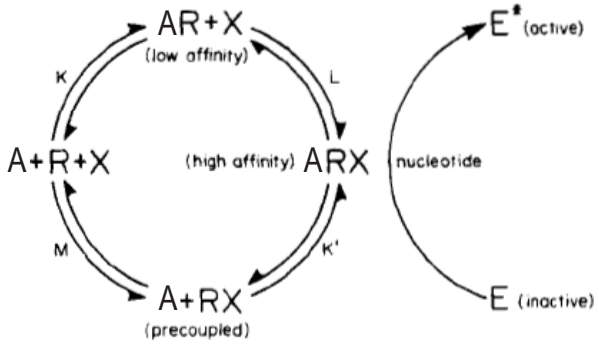
However, several studies suggested that receptors could spontaneously assume the R_a conformation and elicit constitutive signalling activity in the absence of bound ligand. Kjelsberg et al. (1992) identified a single amino acid (alanine 293) in the α_{1B} adrenergic receptor that when substituted for any other amino acid conferred an increase in the basal signalling activity of the receptor and agonist affinity. Further evidence of a wild-type receptor exhibiting constitutive activity was obtained by Tiberi & Caron (1994) with the observation that increasing the expression level of the dopamine 1A and 1B receptors correlated in a linear manner with cellular adenylyl cyclase activity in the absence of agonist. These data suggested that the receptor exists in a dynamic conformational equilibrium between R_i and R_a. The apparent activation of the receptor by an agonist, therefore, would simply reflect a greater affinity for the active conformation and a shift in the equilibrium towards the R_a state (Equation 5). Furthermore, this equilibrium model also accounted for the observed ability of certain ligands to decrease the signalling activity of a receptor below that of the basal level (Chidiac, Hebert, Valiquette, Dennis, & Bouvier, 1994; Costa & Herz, 1989; Costa, Ogino, Munson, Onaran, & Rodbard, 1992; Samama, Pei, Costa, Cotecchia, & Lefkowitz, 1994). Termed *inverse agonists* (Chidiac et al., 1994), these ligands would therefore preferentially stabilize the R_i state of the receptor, shifting the equilibrium of the system towards the inactive receptor reducing receptor activity below that of the unliganded state (Equation 5). In addition, a *neutral antagonist* would be a ligand that binds both R_i and R_a with similar affinity, so as to not significantly shift the equilibrium

from that of the basal, unliganded state and consequently elicit no detectable signalling activity through the receptor.



(Equation 5)

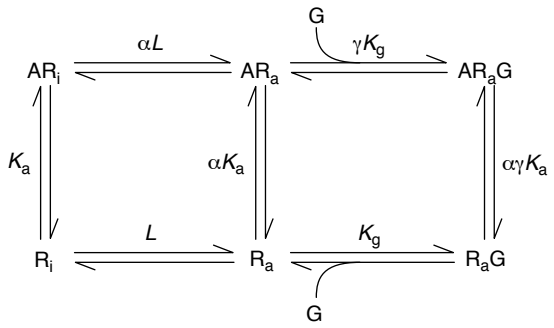
Another significant development on the operational model occurred upon the discovery that certain proteins interacting directly with the receptor could influence its pharmacology through allosteric effects on the receptor itself. The first *proximal effectors* found to exhibit this property were the heterotrimeric G proteins. First indications of this phenomenon were the observations that the addition of guanosine nucleotides could change the affinity of an agonist for a receptor (Kent, De Lean, & Lefkowitz, 1980; Lefkowitz, Mullikin, & Caron, 1976; Maguire, Van Arsdale, & Gilman, 1976; Stadel, DeLean, & Lefkowitz, 1980; Williams & Lefkowitz, 1977). Increasing the amount of GTP or a non-hydrolyzable GTP variant (Gpp(NH)p) was found to shift the competition binding curves of agonists to the right, demonstrating a progressively lower affinity of the receptor for the agonist with increasing concentrations of guanosine nucleotide. Interestingly, this effect was only found for agonists, as the binding curves of antagonists were unaffected. De Lean et al. (1980) proposed the *ternary complex model* to account for the high and low affinity states of agonists, suggesting that the active signalling species of the receptor was a complex between the agonist (A), receptor (R) and some cytoplasmic effector (X) capable of binding GTP:



(Equation 6, Adapted from De Lean, Stadel, and Lefkowitz 1980)

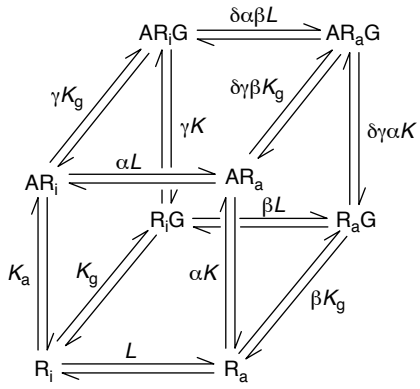
This model proposed that the high affinity state of the receptor was the ternary complex ARX and that binding of a guanosine nucleotide to X would both lead to the activation of downstream effectors (E) and destabilize the high affinity complex. The identity of X, of course, was subsequently discovered to be the heterotrimeric G proteins (reviewed by Limbird, 1981), thus conferring the name G protein-coupled receptors to this family of membrane proteins.

This early model was subsequently refined to synthesize the above observations with the fact that receptors could spontaneously assume active signalling conformations, and thus form the active R_aG protein complex in the absence of ligand. This *extended ternary complex* (ETC) model (Samama, Cotecchia, Costa, & Lefkowitz, 1993) provided a more complete description of the interactions between agonist (A), receptor (R) and G protein (G):



(Equation 7; Kenakin, 2004)

As mentioned above, the ETC model introduced the R_aG species and the rate constant of K_G to describe the affinity of the G protein for the active R_a receptor. The ETC model also incorporates an important aspect of the two-state model with the inclusion of the allosteric constant L, representing the propensity for the receptor to spontaneously assume the active R_a conformation, as well as the parameter α, which describes the differential affinity of a ligand for the inactive versus active state ($[R_i]/[R_a]$) of the receptor. Inverse agonists, therefore, would have an $\alpha < 1$ representing a greater affinity for the inactive receptor while agonists would have an $\alpha > 1$, preferentially stabilizing the active receptor state. Furthermore, the inclusion of the parameter γ allows for a differential affinity of the receptor for G protein when bound to a ligand. A more complex, yet more thermodynamically complete model, the *cubic ternary complex* (CTC) model was subsequently proposed (Weiss, Morgan, Lutz, & Kenakin, 1996), which allowed for the complex between the inactive R_i state and the G protein:



(Equation 8; Kenakin, 2004)

Although the existence of R_iG and AR_iG species in the CTC model was purely theoretical at the time of its proposition, several studies have since demonstrated a pre-coupling of receptors and G proteins (Galés et al., 2005; Hoffmann et al., 2005; Jakubík, Janíčková, Randáková, El-Fakahany, & Doležal, 2011) and provided evidence that inverse agonists can stabilize a non-productive complex between receptors and G proteins (Bouaboula et al., 1997; Brown & Pasternak, 1998; Monczor et al., 2003; Stefan, Overton, & Blumer, 1998; Vásquez & Lewis, 1999), indicating that the comprehensiveness of the CTC model may be

required to provide the most complete description of pharmacological activity for some receptor systems.

3. The pleiotropic nature of GPCR-mediated signalling

1. Promiscuity of G protein coupling and activation

Although early studies of GPCR pharmacology relied on the measurement of a single signalling event that was specific to a G protein subtype activated by a given receptor, it is now known that multiple signalling pathways can be engaged, through a variety of mechanisms, from a single GPCR subtype.

Often, the activation of multiple signalling pathways by a given receptor results from the independent activities of the α- and βγ-subunits of the same activated heterotrimeric G protein. The adenosine A1 receptor, for example, inhibits adenylyl cyclase leading to a decrease in cyclic adenosine monophosphate (cAMP) through the α subunit of G_i proteins; however, the βγ subunits from this heterotrimer, in parallel, activate phospholipase C (PLC) leading to the production of inositol trisphosphate (IP_3) (Tomura et al., 1997). A wide variety of signalling events have since been found to be regulated by βγ subunits (see Table 1) and this mechanism represents one of the most prevalent ways for a receptor to engage multiple signalling events.

Another mechanism leading to the activation of multiple signalling pathways from a single receptor involves the activation of secondary effectors. Instead of resulting from the activation of distinct signalling pathways, in this case the production of one second messenger leads to the downstream production of another. One such example is the Ca^{2+}-dependent production of cAMP through the activation of specific isoforms of adenylyl cyclase (Cali, Zwaagstra, Mons, Cooper, & Krupinski, 1994; Sunahara, Dessauer, & Gilman, 1996). In cells expressing these adenylyl cyclases, GPCRs that couple to an increase in intracellular Ca^{2+}, such as the G_q-coupled muscarinic receptors, can also stimulate the downstream production of cAMP in a G_s-independent manner (Choi, Wong, Hinds, & Storm,

1992). Another such example is the Ca^{2+}-dependent production of arachadonic acid through the activation of phospholipase A2 (Schievella, Regier, Smith, & Lin, 1995).

Although these mechanisms can largely be explained by signal divergence following the activation of a single G protein, the activation of multiple signalling events by a single GPCR can also result from the direct engagement of multiple effectors by the receptor itself. It is now well documented that many GPCRs can promiscuously couple to more than one G protein subtype (reviewed by Hermans, 2003). Often the selectivity of GPCRs for coupling to G proteins occurs at the level of the G protein family and, as a result, many GPCRs can engage multiple members of a given G protein class, such as the adenosine A1 receptor which shows no selectivity between $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, or $G\alpha_{oA}$ (Wise, Sheehan, Rees, Lee, & Milligan, 1999). However, many GPCRs have been shown to couple to multiple G proteins of different families. This represented a fundamental change in our conceptualization of GPCR signalling since traditionally receptors had been classified by the specific G protein family to which they couple. However, many of the muscarinic receptors, including the M1 and M3 subtypes, as well as the angiotensin 2 receptor, have been observed to couple to both G_i and $G_{q/11}$ proteins, leading to both the inhibition of adenylyl cyclase and the stimulation of PLC following activation of a single receptor subtype (Crawford, Frey, & Cote, 1992; Offermanns et al., 1994). Perhaps even more interesting are the examples of GPCRS that couple to both G_s and G_i families of G proteins, since the canonical effects of each are the stimulation and inhibition of adenylyl cyclase, respectively. The α_2 adrenergic receptors (α_2ARs) have been shown to couple to both G_i and G_s in an agonist concentration-dependent manner (Eason, Kurose, Holt, Raymond, & Liggett, 1992; Fraser, Arakawa, McCombie, & Venter, 1989; Jones, Halenda, & Bylund, 1991). By preferentially coupling to G_i at low agonist concentrations and to G_s at higher concentrations, α_2AR agonists produce a bell-shaped concentration-response curve for cAMP production. β_2 adrenergic receptors (β_2AR), on the other hand, undergo a coupling switch from G_s to G_i, following receptor stimulation (Abramson et al., 1988; R. P. Xiao, Ji, & Lakatta, 1995). This promiscuous coupling of this receptor is sequential, whereby the initial activation of G_s leads to the production of cAMP and activation of cAMP-dependent protein kinase A (PKA), which subsequently, through an incompletely understood mechanism, switches the coupling preference of the β_2AR to G_i

(Baillie et al., 2005; Daaka, Luttrell, & Lefkowitz, 1997; E. Stefan et al., 2011). The muscarinic M4 receptor, on the other hand, has been shown to couple to both G_s and $G_{i,}$ leading to the stimulation or inhibiton of cAMP production depending to the isoform of adenylyl cyclase that is co-expressed in the cell (Dittman et al., 1994). Although this promiscuity was originally observed in heterologous systems and was initially interpreted as an artifact of overexpression, the past decade of research has indicated that this phenomenon occurs in physiological contexts and is often the rule rather than the exception (Jin, Wang, & Friedman, 2001; Kilts et al., 2000; Santos-Alvarez & Sánchez-Margalet, 1999; Shi, Wang, Horwitz, & Friedman, 1996; Stanislaus, Ponder, Ji, & Conn, 1998; Table 2).

Table 2: Typical examples of receptors showing multiplicity in G protein coupling.
(Adapted from Hermans, 2003)

Receptor	Gs	Gi	Gq/11	G12	Reference
Adenosine (A3)		x	x		Palmer et al. (1995)
α_2-Adrenergic	x	x			Eason et al. (1992)
β_2-Adrenergic	x	x			Xiao et al. (1995)
Corticotropin-releasing hormone	x	x	x		Grammatopoulos et al. (2001)
Dopamine (D1)	x	x			Jin et al. (2001); Wang et al (1995)
Metabotropic glutamate (1a)	x	x	x		Hermans et al. (2000); Selkirk et al. (2001)
Endothelin (ETB)		x	x		Doi et al. (1999)
Galanin		x	x	x	Wittau et al. (2000)
Glucagon	x	x			Kilts et al. (2000)
Gonadotrophin releasing hormone	x	x	x		Stanislaus et al. (1998)
Histamine H2	x		x		Kühn et al. (1996)
Luteinizing hormone	x	x	x		Herrlich et al. (1996); Kühn and Gudermann (1999)
Melatonin		x	x		Brydon et al. (1999)
Muscarinic (M1 and M3)		x	x		Akam et al. (2001); Offermanns et al. (1994)
Muscarinic (M4)	x	x			Dittman et al. (1994)
Neurotensin		x	x		Grisshammer et al. (2001)
Pancreastin		x	x		Santos-Alvarez et al. (1999)
Parathyroid hormone	x		x		Offermanns et al. (1996)
Platelet-activating factor		x	x		Shi et al. (1996)
Prostacyclin	x	x	x		Lawler et al. (2001)
Prostaglandin (EP3D)	x	x			Negishi et al. (1995)
Serotonin (5-HT_{2C})		x	x		Cussac et al. (2002)
Sphingosine 1-phosphate (Edg3)	x		x	x	Windh et al. (1999)
Substance P			x	x	Barr et al. (1997)
Thyrotropin	x	x	x	x	Allgeier et al. (1997); Allgeier et al. (1994)
Thrombin		x	x	x	Barr et al. (1997); Ogino et al. (1996)
Vasopressin V1a		x	x		Abel et al. (2000)
Vasoactive intestinal peptide	x	x			Berrada et al. (2000); Luo et al. (1999)

2. Non-G protein effectors

In addition to heterotrimeric G proteins, GPCRs have also been shown to interact with a host of other proteins that control receptor signalling activity and its regulation (reviewed by Bockaert et al. 2004; Ritter & Hall, 2009). While many of these proteins had previously been identified to participate only in the regulation of G protein-dependent signalling, more recent evidence suggests that they can also elicit *bona fide* signalling responses themselves in a G protein-independent manner. The common attribute of these GPCR interacting proteins (GIPs) is that they interact with receptors, usually through contacts with their intracellular loops or cytoplasmic C-terminal tail, yet they participate in GPCR-mediated signalling in a number of diverse ways.

i. β-arrestin

One of the most characterized of all non-G protein effectors is the multifunctional β-arrestin protein. First discovered in the retina, the "visual" arrestins were identified as proteins that bound to activated rhodopsin to desensitize these light-activated GPCRs (Pfister et al., 1985). Outside of the retina, the nonvisual arrestins (β-arrestin 1 and 2) were later identified to play a homologous role in the desensitization of non-rhodopsin GPCRs (Attramadal et al., 1992; Benovic et al., 1987; Lohse, Benovic, Codina, Caron, & Lefkowitz, 1990). While first identified to desensitize GPCR signalling by preventing receptor: G protein coupling through steric hindrance (Attramadal et al., 1992), these proteins were subsequently found to also play a key role in the subsequent endocytosis of receptors (Zhang, Ferguson, Barak, Ménard, & Caron, 1996). In this well-conserved mechanism, β-arrestin act as a molecular scaffold, linking activated receptors to the endocytic machinery of the cell, interacting directly with the clathrin adaptor protein AP-2 (Goodman et al., 1996; Laporte et al., 1999). Thus, β-arrestin attenuates receptor signalling activity by facilitating the endocytosis of the activated receptor, after which it is either trafficked to lysozomes to be degraded or is recycled back to the cell surface (Ferguson et al., 1996). β-arrestin has also been shown to play a role in determining this post-endocytic fate of the receptor by regulating its ubiquination. Through interactions with the E3 ubiquitin ligases Mdm2 (Shenoy et al. 2001), Nedd4 (Shenoy et al. 2008) and AIP4 (Marchese et al., 2003), β-

arrestin promotes receptor ubiquitination, which regulates the trafficking of receptors following internalization. Interestingly, the dynamics of the ubiquitination of β-arrestin itself also seems to play a role in the determination of the post-endoctyic fate of receptors (Shenoy & Lefkowitz, 2003).

Interestingly, recent evidence has indicated that internalized receptors may continue to signal from the cytoplasm, through both G protein-dependent and -independent mechanisms. Several recent studies have found evidence of cAMP production promoted by receptors localized in post-endocytic vesicles, including the type 1 parathyroid hormone receptor (PTH1R) (Ferrandon et al., 2009), the pituitary adenylate cyclase activating polypeptide (PACAP) receptor (Merriam et al., 2013) and the β_2AR (Irannejad et al., 2013). In this latter study, although both activated receptors and G proteins were found to co-localize in endosomes, neither β-arrestin nor clathrin were detected in these vesicles. The authors propose that the receptor- and agonist-dependent G_s coupling and cAMP formation observed from endosomes occurs following the uncoating of clathrin from the vesicles. This surprising finding that an event believed to function in the arrest of GPCR signalling could initiate additional signalling events demonstrates that what were previously believed to be desensitization mechanisms might actually represent the initiation of a second wave of GPCR signalling.

In addition, β-arrestin has been found to participate directly in the activation of signalling pathways following receptor stimulation by scaffolding receptors to various effectors (DeWire, Ahn, Lefkowitz, & Shenoy, 2007; McDonald & Lefkowitz, 2001; Reiter & Lefkowitz, n.d.; Shukla, Xiao, & Lefkowitz, 2011). This mechanism is well documented in the activation of certain members of the mitogen activated protein kinase (MAPK) family by many GPCRs. Interestingly, depending on the receptor that is stimulated, different mechanisms have been proposed to underlie this β-arrestin-mediated MAPK activation. In some cases, β-arrestin acts to scaffold members of the ERK1/2 or JNK3 MAPK cascade to receptor complexes in internalized vesicles to facilitate activation of the pathway (DeFea et al. 2000; McDonald et al. 2000; Luttrell et al. 2001; DeFea et al. 2000). While classical MAPK pathway activated by receptor tyrosine kinases (RTKs) often leads to the nuclear translocation of

MAPK, GPCRs that activate MAPK in these cytoplasmic vesicles have often been shown to restrain MAPK signalling to the phosphorylation of predominantly cytoplasmic targets (Ge, Ly, Hollenberg, & DeFea, 2003; Luttrell et al., 2001; Tohgo, Pierce, Choy, Lefkowitz, & Luttrell, 2002). Another common mechanism of MAPK activation by GPCRs involves the transactivation of an RTK. Interestingly, β-arrestin has been shown to participate in this mode of MAPK activation in several distinct ways. In the case of the β_1AR, agonist-dependent scaffolding of the tyrosine kinase c-Src by β-arrestin participates in the metalloproteinase-dependent cleavage of an extracellular ligand for the epidermal growth factor receptor (EGFR), leading to the activation of ERK1/2 (Noma et al., 2007). Such RTK transactivation pathways have similarly been identified for a number of GPCRs, including angiotensin receptors (Eguchi et al., 1998), M1 muscarinic receptor (Prenzel et al., 1999) and lysophospholipid receptors (Prenzel et al., 1999). Intriguingly, the V2 vasopressin receptor (V2R), which has been shown to activate ERK1/2 through the transactivation of insulin-like growth factor receptor (IGFR), utilizes β-arrestin in a slightly different way. Whereas c-Src was found to play a role upstream of the release of an extracellular ligand for the IGFR, β-arrestin was found to act downstream of the transactivated IGFR (Oligny-Longpré et al., 2012).

β-arrestin has also been observed to participate in the regulation of a number of other important signalling events following GPCR activation, including the regulation of the phosphatidylinositol-3-kinase (PI_3K)/Akt/glycogen synthase kinase 3 (GSK3)/protein phosphatase 2A (PP2A) pathway (Beaulieu et al., 2005, 2008; Goel, Phillips-Mason, Raben, & Baldassare, 2002; Lu, Su, Liu, & Daskalakis, 2012; Naga Prasad et al., 2002), the NFκB pathway (Gao et al., 2004; Witherow, Garrison, Miller, & Lefkowitz, 2004), phosphodiesterase 4D5 (PDE4D5) (Baillie et al., 2005), filamin A (Scott et al. 2006), phosphatase and tensin homolog (PTEN) (Lima-Fernandes et al., 2011), LIM kinase/cofilin (Zoudilova et al., 2007) and RhoA (Barnes et al. 2005). β-arrestin has also been shown to regulate transcriptional activity directly following activation and translocation to the nucleus by interacting with transcription factors (Hoeppner, Cheng, & Ye, 2012; Zhuang, Hu, Xin, Zhao, & Pei, 2011), co-activators (Kang et al., 2005; Shenoy et al., 2012) or co-

repressors (Mo et al., 2008) at the promoter regions of genes, such as p27 and c-fos (Kang et al., 2005).

ii. G protein-coupled receptor kinases

GPCRs also interact with various classes of kinases that serve diverse roles in the regulation of receptor signalling activity. One such class is the G protein-coupled receptor kinases (GRKs), a family of seven serine/threonine kinases that preferentially phosphorylate activated GPCRs (Benovic et al., 1987; Penela, Ribas, & Mayor, 2003). The primary role of these kinases is to phosphorylate and desensitize receptors following agonist stimulation. The phosphorylation of sites on the intracellular loops or carboxy terminal tail of receptors increase its affinity for β-arrestin and, thus, promote its subsequent internalization (Bouvier et al., 1988; J. A. Pitcher, Freedman, & Lefkowitz, 1998). In addition to their role in the desensitization of receptors, GRKs themselves are known to modulate GPCR signalling through the regulation of other effectors including $G\alpha_q$ (Carman, 1999), $G\beta\gamma$ (Eichmann et al., 2003; Koch, Inglese, Stone, & Lefkowitz, 1993), Raf kinase inhibitor protein (RKIP) (Lorenz, Lohse, & Quitterer, 2003), MEK (Jiménez-Sainz et al., 2006), PI_3K (Naga Prasad et al., 2002) and Akt (Liu et al. 2005).

iii. Second messenger kinases

Second messenger kinases such as PKA and Ca^{2+}-dependent protein kinase C (PKC), in addition to their key roles in propagating signals downstream of G_s and G_q, respectively, also participate in the desensitization of GPCRs following receptor stimulation through the phosphorylation of specific serine and threonine residues in receptor intracellular loops and carboxy terminal tail (Benovic et al., 1985; Bouvier, Leeb-Lundberg, Benovic, Caron, & Lefkowitz, 1987; Klein, Sullivan, Skorupa, & Aguilar, 1989; Leeb-Lundberg, Cotecchia, DeBlasi, Caron, & Lefkowitz, 1987). Since the activity these kinases is dependent on the production of their second messenger regulators, they provide a elegant means of negative feedback on the receptors initiating these signalling events. Through the phosphorylation of conserved sequences in these regions, PKA and PKC introduce a highly charged moiety that decreases the ability of the receptor to interact with and activate G proteins (Hausdorff, Caron, & Lefkowitz, 1990). These phosphorylation sites are distinct from those

targeted by GRKs and generally do not promote the recruitment of β-arrestin nor receptor endocytosis (Pitcher et al. 1992). More recently, the ERK/MAPK effector p90 ribosomal S6 kinase 2 (RSK2) has been shown to phosphorylate the 5-hydroxytryptamine 2A (5-HT_{2A}) receptor leading to an attenuation of its signalling (Strachan, Allen, Sheffler, & Roth, 2010), implicating yet another downstream effector in a negative regulatory mechanism to limit GPCR signalling.

iv. Src family kinases

Src family kinases are non-receptor tyrosine kinases that play a role in a variety of cellular events including proliferation, survival, differentiation, adhesion, migration and cell cycle control (Sun, McGarrigle, and Huang 2007; Miller and Lefkowitz 2001). The prototypical member of this family, c-Src, has been implicated in a number of mechanisms that regulate signalling at GPCRs, including its previously described role in the activation of MAPK by various GPCRs (*Section 3.2.i;* Cao et al., 2000; DeFea, Vaughn, et al., 2000; Luttrell et al., 1999). c-Src also participates in a signalling complex with the angiotensin 2 receptor and Src homology phosphatases 1 and 2 (SHP-1 and SHP-2) (Alvarez, Seguin, Villarreal, Nahmias, & Ciuffo, 2008) to regulate the activity of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Marrero, Venema, Ju, Eaton, & Venema, 1998) and focal adhesion kinase (FAK) (Seguin, Villarreal, & Ciuffo, 2012). c-Src has also been shown to phosphorylate several components of the cellular endocytic machinery, including dynamin and clathrin, to promote receptor internalization (Miller 2000; Ahn 1999). Other Src family kinase members also participate in the regulation of GPCR signalling. Angiotensin receptors have been shown to modulate the activity of the calcium-sensitive tyrosine kinase Pyk2 through the activation of the Src family kinase member Yes (Tang et al. 2000). The Src family member Fyn interacts directly with the 5-HT_6 receptor to promote activation of ERK1/2 in a β-arrestin-independent manner (Yun et al., 2007). Yet another Src family member, Hck, is recruited to the chemokine CXCR1 receptor to participate in the release of neutrophil granules (Barlic et al., 2000). Through these diverse mechanisms, the multifunctional Src family kinases have become recognized as highly important regulators of GPCR signalling.

v. GPRC scaffolding proteins

Accumulating evidence suggests that GPCRs and their effectors do not signal through simple diffusion mechanisms but rather that receptors are part of higher order assemblies or *signalosomes.* These complexes often contain the necessary effectors and regulatory molecules of a given signalling module to ensure a rapid and efficient response to extracellular stimuli. Often these signalosomes are nucleated by multi-domain scaffolding proteins that can interact, often dynamically, with multiple effector molecules. Several classes of scaffolds have been identified that assemble proteins from a diverse array of signalling pathways. One such scaffolding protein is the previously discussed β-arrestin, which has been shown to interact with a host of signalling proteins including casein kinase II, Yes, PI_3K, diacylglycerol kinases, PP2A and PDE4E (Xiao et al., 2007), as well as many of the effectors of the ERK1/2 (MEK1/ERK1/2) (DeFea et al. 2000) and JNK3 (ASK1/MAPK4/JNK3) pathways (McDonald et al., 2000), and several of the proteins that participate in receptor endocytosis (clathrin/AP-2/ARF6) (Claing et al., 2001; Goodman et al., 1996; Laporte et al., 1999).

Many other scaffolds interact with the carboxy terminal tails of GPCRs through specialized PSD-95/Discs-large/ZO-1 (PDZ) homology domains. The β_1 adrenergic receptor (β_1AR) has been shown to interact with several PDZ proteins, including membrane-associated guanylate kinase-like protein inverted-2 (MAGI-2) (Xu et al. 2001) and postsynaptic density protein 95 (PSD-95) (Hu et al., 2000), among others (He et al., 2006). This interaction with PSD-95 acts as a scaffold to promote β_1AR-mediated regulation of the N-methyl-D-aspartate (NMDA) class of glutamate receptors (Hu et al., 2000). The β_2AR and the kappa opioid receptor, on the other hand, interact with the PDZ-containing scaffold Na^+/H^+ exchanger regulatory factors 1 and 2 (NHERF1/2), to regulate the activity of Na^+/H^+ exchanger type 3 (NHE3) (Hall et al. 1998; Hall et al. 1998; Huang et al. 2004). The metabotropic glutamate family of receptors (mGluRs) have also been shown to interact with several PDZ-containing scaffolds, including Shank (Tu et al., 1999), tamalin (Kitano et al., 2002) and PICK1 (Boudin et al., 2000). In addition to these examples, many more PDZ-containing scaffolds have been identified that interact with other GPCRs including dopamine D2 and D3 receptors, somatostatin 5 receptor, human leutinizing hormone receptor, vasoactive intestinal

polypeptide receptor 1, lysophosphatidic acid receptor 2, 5-HT receptors 2A and 2C, parathyroid hormone receptor 1 and the bradykinin receptor (reviewed in Magalhaes, Dunn, & Ferguson, 2012).

Several other non-PDZ-containing scaffolds have also been identified to bind to and regulate GPCR signalling. Several members of the A-kinase anchoring protein (AKAP) family of scaffolds also interact with GPCRs (Malbon, Tao, & Wang, 2004). The AKAP5 (also known as AKAP79) protein, for example, scaffolds the β_2AR to several important effectors of cAMP pathway, including the PKA regulatory subunits RI and RII, the phosphatase PP2B and PKC (Wang et al. 2006). Interestingly, this AKAP-scaffolded complex has been shown to play a role in the G_s to G_i coupling switch previously described (Lynch et al., 2005). In this mechanism, cAMP promotes receptor phosphorylation by the AKAP-scaffolded PKA, switching the coupling preference of the β_2AR to G_i. The AKAP-scaffolded PP2B and the β-arrestin-dependent recruitment of the phosphodiesterase PDE4D5 act to spatially constrain this activity to the vicinity of the activated β_2AR (Lynch et al., 2005), altogether ensuring a rapid, localized and regulated response. Other interesting scaffolding proteins regulating GPCR activity include the Homer proteins, which link mGluRs to various signalling molecules including IP_3 receptors (Tu et al., 1998), syntaxin (Minakami, Kato, & Sugiyama, 2000) and Shank (Tu et al., 1999).

vi. Other effectors

Another important group of effectors regulating GPCR signalling are the appropriately named regulators of G protein signalling (RGS) proteins. This is a large family of proteins grouped into eight distinct classes based on sequence homology (Ladds & Willars, 2006), all of which containing a GTPase activating domain to serve their primary role in terminating G protein signalling by accelerating the hydrolysis of the GTP nucleotide bound to the active Gα subunit (De Vries, Zheng, Fischer, Elenko, & Farquhar, 2000). RGS proteins also exhibit an antagonistic effect on G protein signalling by physically blocking their interaction with downstream effectors (Hepler, Berman, Gilman, & Kozasa, 1997). These proteins have an N-terminal membrane targeting signal which enables them to localize in close proximity to their targets, the G proteins, and in some cases RGS proteins have been found to directly

interact with the third intracellular loop of GPCRs, including the M1 muscarinic receptor (Bernstein et al., 2004) and the α_{1A} adrenergic receptor (Hague et al., 2005). In addition to their GTPase domains, RGS proteins consist of several other domains important for protein-protein interactions, including PDZ domains, GoLoco motifs, G protein gamma-like (GGL) domains and phosphotyrosine binding (PTB) domains, all of which can be used to interact with other effectors to further regulate GPCR signalling beyond their inhibitory effect on G proteins (Magalhaes et al., 2012).

Many members of the small GTP-binding protein superfamily are also regulated by GPCRs, often through direct interactions. These "small G proteins" are classified into five functional classes and regulate a wide variety of cellular events including modulation of actin cytoskeleton, proliferation, growth, survival, endocytosis, vesicular trafficking and gene transcription, among a host of other functions (Takai, Sasaki, & Matozaki, 2001). One of the most characterized of these relationships is between $G_{12/13}$-coupled GPCRs and the Rho family of small G proteins, which play an important role in various cytoskeletal remodeling events (Sah, Seasholtz, Sagi, & Brown, 2000). These receptors activate Rho through direct interactions between activated $G_{12/13}$ proteins and the Rho guanosine exchange factor (RhoGEF) family of proteins (e.g. PDZ-RhoGEF, LARG and p115RhoGEF), which stimulate the exchange of GDP for GTP to activate Rho (Vogt, Grosse, Schultz, & Offermanns, 2003). GPCRs also interact with many members of the Rab family, which regulate the vesicular trafficking of receptors to and from the plasma membrane (Magalhaes et al., 2012). In addition, several GPCRs have been shown to modulate the activity of Ras family GTPases; however, these interactions are often indirect and occur through other effectors. Recent studies, for example, have shown that several heterotrimeric G proteins can modulate the activity of Ras through the tetratricopeptide repeat 1 (TPR1) protein following GPCR activation to potentiate the Ras-dependent phosphorylation of downstream targets such as ERK1/2 and IκB (Liu et al. 2010; Kwan et al. 2012). Arf GTPases, on the other hand, have been observed to directly interact with several GPCRs to regulate their endocytosis, including the β_2AR, angiotensin 1 receptor, the endothelin type B receptor and the V2R (Claing et al., 2001; Houndolo, Boulay, & Claing, 2005).

GPCRs have also been shown to regulate the activity of a number of ion channels and ionotropic receptors. Although many of these signalling events occur indirectly through other signalling intermediates, such as the Gα or βγ subunits (Altier, 2012; Rojas & Dingledine, 2013), several GPCRs have been shown to physically interact with and modulate ion channel activity. D1 and D5 dopamine receptors, for example, interact directly with NMDA (Lee et al., 2002) and γ-aminobutyric acid type A ($GABA_A$) (Liu et al. 2000) ionotropic receptors, respectively, to control their activity and subsequent coupling to downstream effectors. The metabotropic glutamate receptor mGluR1a, when expressed in Purkinje neurons, physically interact with the voltage-sensitive CaV2.1 Ca^{2+} channel via its C-terminal tail (Kitano et al., 2003) to provide spatiotemporal regulation of intracellular Ca^{2+} upon glutaminergic stimulation. The nociception ORL1 receptors have been observed to regulate N-type CaV2.2 Ca^{2+} channels via an interaction with their carboxy terminal tail (Beedle et al., 2004).

In addition to those described above, a number of other proteins have been found to interact with GPCRs to regulate their signalling, including calmodulin (Wang, Surratt, and Sadée 2002; Wang, Sadée, and Quillan 1999), receptor activity modifying proteins (RAMPs) (Hay, Poyner, & Sexton, 2006), spinophilin (Smith, Oxford, and Milgram 1999; Richman et al. 2001; Charlton et al. 2008), PP2A (Krueger, Daaka, Pitcher, & Lefkowitz, 1997; Mao et al., 2005), Src homology 3 domain containing proteins such as Grb2, Nck (Oldenhof et al., 1998) and endophilin (Tang et al. 1999).

Taken together, the above discussion clearly emphasize the vast diversity of GPCR signaling beyond the classical G protein-mediated regulation of canonical second messengers such as cAMP, Ca^{2+} and diacylglycerol. This diversity is only starting to become recognized for its impact on molecular pharmacology and is rarely taken into consideration in the context of drug discovery.

4. Functional selectivity

1. Introduction to functional selectivity

The previous two decades of research has revealed that GPCR signalling is far more extensive than the simple coupling of a receptor to a single G protein to stimulate the production of a specific second messenger. As described above, we now know that many GPCRs are capable of engaging multiple G proteins, often from different families, and sometimes in a sequential or agonist concentration-dependent manner. Furthermore, the discovery that GPCRs can couple to other non-G protein effectors revealed that additional G protein-independent signalling events contribute to the receptor's complete signalling repertoire. In the context of the models of receptor theory described to this point, such observations do not necessarily negate their applicability but may necessitate adjustments to account for the extensive signalling diversity that has been observed. The CTC model (Equation 8), for example, describes the production of a single agonist-receptor-G protein ($A \cdot R_A \cdot G$) ternary complex that elicits the receptor signalling response. If the conformation of the active receptor species is the same regardless of the effector considered, whether it be a different G protein or a non-G protein effector initiating a signalling response, the CTC model would still provide a comprehensive description of GPCR activation. One could simply replace the G protein (G) with a general effector (E) and the structure and equilibrium dissociation constants of the model would remain the same. Since signalling would be engaged by the same active receptor conformation, the pharmacology of a ligand should remain identical regardless of the pathway being measured.

However, soon after the discovery that receptors could couple to multiple pathways, many researchers began to independently observe that the pharmacology of a ligand towards one signalling pathway is not always conserved for all others engaged by a given receptor. To date, numerous studies have found that both the efficacy and potency of a ligand can differ from one pathway to another. Such pathway-dependent pharmacology has been shown to occur in various signalling contexts: in cases where a receptor engages multiple pathways though promiscuous coupling to G proteins, in G protein-dependent vs. –independent signalling events, and even a functional dissociation between signalling and receptor

desensitization mechanisms (see *Annex II*, page xviii; Stallaert, Christopoulos, and Bouvier 2011; Kenakin 1995; Urban et al. 2007; Kenakin 2010; Galandrin, Oligny-Longpré, and Bouvier 2007). These data strongly suggest that agonists do not simply stabilize a single, active receptor conformation, but instead may exhibit a selectivity for multiple active signalling states, with differing abilities to engage effectors. Collectively, this phenomenon has been given several names, including *agonist directed trafficking of stimulus* (Kenakin 1995), *biased agonism* (Jarpe et al., 1998), as well as, more generally, *functional selectivity* (Stallaert et al., 2011; Urban et al., 2007).

2. Biased agonism at G protein-dependent signalling pathways

One of the earliest reports of ligand functional selectivity described the differential abilities of α_2AR ligands to couple the receptor to G_i and G_s proteins (Eason, Jacinto, & Liggett, 1994). The researchers found that among the six ligands evaluated, all of them coupled to G_i as full agonists, yet the efficacies towards G_s differed greatly among the ligands, with some ligands exhibiting no activity whatsoever. Although these results suggest that ligands exhibit functional selectivity in G protein coupling, this study demonstrates a relatively common ambiguity in many studies claiming biased signalling, What looks like functional selectivity can sometimes result instead from differences in the strength or amplification of effector pathways coupled to a given receptor. In the above case, differences in G protein coupling could be explained by disparity in the ability of ligands to stabilize a single active receptor state that has a greater affinity for G_i vs. G_s. Ligands showing selective coupling of the receptor to G_i could simply be exhibiting partial agonism, stabilizing less of the active receptor that preferentially couples to G_i over G_s (Kenakin 1995). Other examples of this phenomenon include cases where the signalling events being measured are subject to different levels of amplification. In such cases, differences in agonist efficacy would appear much larger in less amplified pathways giving the appearance of biased signalling among a set of ligands that simply differ in their intrinsic efficacy to stabilize a single active state.

More direct evidence of ligand bias would be a change in the rank-order of ligand efficacy towards distinct signalling events. In another pioneering study, Berg et al. (1998) assessed the ability of ligands to stimulate the G_q-mediated production of inositol phosphates (IP)

and the G_{13}-mediated accumulation arachadonic acid (AA) through the 5-HT_{2A} and 5-HT_{2C} receptors. This study was particularly elegant in its design since the activation of both pathways was measured simultaneously, through the production of [^{3}H]IP and [^{14}C]AA, thus eliminating any potential influence of different assay conditions on the results obtained. In this study, researchers found that the rank order of efficacy was considerably different for each of the pathways tested. At 5-HT_{2C} receptors, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, bufotenin and lysergic diethylamide acid showed greater relative efficacy towards AA accumulation while 3-trifluoromethylphenyl-piperazine and quipazine were more efficacious towards IP production. Unlike the previous example, such differences in the rank-order of ligand efficacy cannot be explained simply by differences in pathway amplification and thus provide strong evidence of functional selectivity of 5-HT receptor ligands to stabilize receptor conformations with different G protein coupling preferences.

In addition to changes in the rank-order of ligand efficacies for distinct signalling pathways, differences in the rank-order of potencies have also been reported. In fact, perhaps the first unequivocal evidence of functional selectivity was the observation that two endogenous peptide ligands of the PACAP receptor exhibit reversals in their potency rank order towards two distinct pathways (Spengler et al., 1993). Whereas $PACAP_{1-27}$ is more potent at stimulating G_s-dependent cAMP production, $PACAP_{1-38}$ is a more potent activator of G_q-dependent IP production. A similar reversal in potency rank-order was observed for ligands of the *Drosophila* octopamine-tyramine receptor heterologously expressed in Chinese hamster ovary (CHO) cells (Robb et al., 1994).

All of the studies cited thus far demonstrate ligand bias in G protein coupling indirectly, through measurements of second messengers downstream of G protein activation. Chakrabarti et al. (1995), however, provided the first evidence of the functional selectivity of μ-opioid receptor ligands for G proteins directly. Through measurements of α-azidoanilido[^{32}P]GTP photoaffinity labeling of four distinct Gα subunits of the $G_{i/o}$ family, these researchers found differences in the potency of ligands to couple the receptor to

distinct G protein subtypes, providing more direct evidence that ligands can exhibit a selectivity towards distinct G protein-coupled states of the receptor.

In the two decades that have passed since these seminal studies, numerous other GPCRs have been observed to exhibit ligand functional selectivity at the level of G protein coupling, including bombesin receptors (MacKinnon, Waters, Jodrell, Haslett, & Sethi, 2001), parathyroid hormone receptors (Takasu, Gardella, Luck, Potts, & Bringhurst, 1999), follicle-stimulating hormone receptors (Arey, 1997), tachykinin receptors (Palanche et al., 2001), adenosine A1 receptors (Cordeaux et al., 2000), β adrenergic receptors (Wenzel-Seifert & Seifert, 2000), calcitonin receptors (Watson et al., 2000), cannabinoid receptors (Bonhaus, Chang, Kwan, & Martin, 1998), dopamine receptors (Cordeaux, Nickolls, Flood, Graber, & Strange, 2001), muscarinic receptors (Akam et al., 2001) and prostanoid receptors (Negishi et al., 1995), among many others.

3. G protein-dependent vs. -independent signalling bias

Soon after the discovery that GPCRs could couple to and activate additional signalling events through non-G protein effectors, various instances of biased signalling were reported comparing G protein-dependent versus G protein-independent signalling events elicited from the same receptor. Many of these examples involve the previously described β-arrestin protein (Shukla et al., 2011). Interestingly, in many such cases of G protein-dependent vs. β-arrestin-dependent signalling bias, ligands show a more extreme selectivity, often exhibiting agonism towards one pathway and a complete lack of activity towards the other. Several peptide ligands for the angiotensin 1A receptor ($AT_{1A}R$), for example, exhibit a very clear bias towards β-arrestin-dependent signalling. These ligands, including TRV120027 (Violin et al., 2010), SII ((Sar^{1}, Ile^{4}, Ile^{8})-angiotensin II) (Wei et al., 2003), SI ((Sar^{1}, Ile^{8})-angiotensin II) and DVG ((Asp^{1}, Val^{5}, Gly^{8})-angiotensin II) (Zimmerman et al., 2012) promote the engagement of various β-arrestin-dependent signalling events, including the activation of ERK1/2, yet possess no efficacy for the activation of G protein-dependent signalling at the receptor. Even more striking is the bias observed for some “beta-blocker” compounds targeting the β-adrenergic receptors, which exhibit a “reversal” in efficacy toward two distinct pathways. Propranolol, for example,

behaves as an inverse agonist towards G_s-dependent cAMP production at the β_2AR, yet it was found to act as an agonist towards β-arrestin-dependent ERK1/2 activation (Azzi et al., 2003). This important finding firmly established that receptors could assume multiple active signalling states with distinct abilities to engage effectors since the conformation(s) stabilized by propranolol could engage β-arrestin-dependent signalling yet prevented a functional coupling to G proteins. This phenomenon was later observed for other GPCRs, including the closely related β_1AR (Galandrin and Bouvier 2006), the 5-HT_{2C} (Werry, Gregory, Sexton, & Christopoulos, 2005) and the delta opioid receptor (Audet et al. 2005).

An additional level of signalling "texture" has been observed for the PTH1R, which can couple to G_s, G_q and β-arrestin to elicit a variety of signalling events. Whereas the endogenous peptide ligands of the receptor, parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) are able to activate signalling through all three of these effectors, certain modifications of PTHrP ($PTHrP_{2-36}$ and Bpa^1-PTHrP) were found to abolish the peptides' ability to engage G_q and β-arrestin, while G_s-dependent cAMP production remained intact (Bisello et al., 2002). In a later study, another group observed that the (D-Trp^{12}, Tyr^{34})-PTH_{7-34} peptide, an inverse agonist for G_s-dependent signalling, retained the ability to recruit β-arrestin to the receptor (Gesty-Palmer et al., 2006). Therefore, ligands for the PTH1R can stabilize multiple signalling conformations of the receptor, including those biased to either G protein-dependent or β-arrestin-dependent signalling. It is unclear at this point if unbiased ligands (i.e. those that can couple the receptor to G_s, G_q and β-arrestin) stabilize a single receptor conformation that can couple to all three effectors or if these ligands instead stabilize multiple conformations that selectively couple to each of the effectors. This concept will be expanded upon in *Section 5: Conformational basis of functional selectivity* (page 35).

4. Uncoupling of signalling from receptor desensitization

Another related and important consequence of ligand-selective receptor conformations is the reported ability of some compounds to differentially modulate the desensitization of receptor activity. In parallel to the engagement of signalling pathways, receptor stimulation initiates several mechanisms that provide negative feedback on receptor activity, often

through the phosphorylation of intracellular loops or the carboxy terminal tail of the receptor to decouple it from effectors and promote receptor endocytosis (Kelly, Bailey, & Henderson, 2008). However, there have been several reports of ligands exhibiting an uncoupling of these desensitization mechanisms from receptor-promoted signalling, often by modulating the interaction between the receptor and GRKs or second messenger kinases to change or even prevent receptor phosphorylation (Kelly et al., 2008). The lack of correlation between agonist efficacy and rate of desensitization has been observed for many GPCRs including the CB1 cannabinoid receptor (Luk et al., 2004), the β_2AR (Moore et al., 2007), the 5-HT_{2C} receptor (Stout, Clarke, & Berg, 2002), the α_1 adrenergic receptor (Akinaga et al., 2013), the $AT_{1A}R$ (Thomas, Qian, Chang, & Karnik, 2000), the neuropeptide Y1 receptor (Pheng et al., 2003), the cholecystokinin receptor (Roettger et al., 1997), the PTHR1 (Bisello et al., 2002) and the CCR7 chemokine receptor (Kohout et al., 2004). Most extremely, the platelet-activating factor (PAF) receptor has even been shown to be phosphorylated and internalized by the inverse agonist WEB2086, through a mechanism distinct from what is observed following treatment with the endogenous PAF agonist (Dupré et al., 2007).

This phenomenon has been studied extensively in the opioid receptor system, where it was coined as relative activity versus endocytosis ("RAVE") (Whistler, Chuang, Chu, Jan, & von Zastrow, 1999). The μ-opioid receptor (μOR) agonists [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin (DAMGO) and morphine have been shown in many cell types to undergo distinct mechanisms of desensitization, mediated primarily through GRKs and PKC, respectively (Johnson et al., 2006; Whistler & von Zastrow, 1998). These data suggest that, despite both behaving as agonists at the μOR, DAMGO and morphine stabilize distinct conformations of the receptor that differ in their recognition by kinases. These results are somewhat controversial, as it seems that in certain cellular contexts these desensitization mechanisms are not so clear-cut. For example, although morphine does not promote β-arrestin recruitment in cultured cells, β-arrestin knockout mice fail to display antinociceptive tolerance (Groer et al., 2007) and experience enhanced reward behaviours (Bohn et al., 2003), indicating a role for β-arrestin in the *in vivo* response to the drug. Furthermore, upon overexpression of GRK2 in human embryonic kidney-293 (HEK293)

cells, morphine exhibits the ability to recruit β-arrestin and induce receptor internalization (Bohn et al. 2004). Thus, although the receptor conformation stabilized by morphine is a poor substrate for GRK phosphorylation, in tissues where GRK expression is elevated it may contribute to receptor desensitization. Furthermore, in some tissues morphine-induced desensitization requires additional PKC activation, such as through the stimulation of a G_q-couple receptor (Bailey, Kelly, & Henderson, 2004). Altogether, these data suggest that although DAMGO and morphine may stabilize receptor conformations that differ in their susceptibility to phosphorylation by GRKs and PKC, tissue-specific expression/activity/localization of these effectors may ultimately determine the mechanisms through which desensitization occurs for each agonist (Haberstock-Debic, Kim, Yu, & von Zastrow, 2005). Interestingly, yet another μOR agonist, herkinorin, does not promote either PKC- nor GRK-induced desensitization (Groer et al., 2007), perhaps by stabilizing yet another active signalling conformation of the μOR.

Differences in the recruitment of kinases following receptor activation ultimately leads to changes in the phosphorylation pattern of the receptor. Indeed, differences in μOR phosphorylation have been observed between morphine and another μOR agonist, etorphine (Zhang 1998). This study demonstrated that, in contast to etorphine, morphine did not induce receptor phosphorylation, β-arrestin recruitment or receptor internalization unless GRK2 was overexpressed. Similar dissociations between agonist activity and receptor phosphorylation have been shown for many other receptors, including the β_2AR, where different agonists were found to induce distinct patterns of phosphorylation (Trester-Zedlitz, Burlingame, Kobilka, & von Zastrow, 2005). These differences were isolated in the proximal part of the carboxy terminal tail of the β_2AR, which has been shown to assume different conformations depending on the agonist (Granier et al., 2007). Such distinct, agonist-specific phosphorylation patterns confer different avidities for effectors, such as β-arrestin, which interact with the receptor in a phosphorylation-dependent manner, providing yet another means for biased signalling at GPCRs. For example, Nobles et al. (2011) found that isoproterenol and carvedilol, two ligands with distinct signalling profiles at the β_2AR, promoted distinct receptor phosphorylation patterns through the recruitment of different GRKs. Whereas isoproterenol promoted the recruitment of both

GRK2 and GRK6, carvedilol stabilized a receptor conformation phosphorylated only by GRK6. While both ligands promoted the recruitment of β-arrestin, these distinct phosphorylation patterns or "barcodes" induced differences in the conformation of bound β-arrestin and, consequently, differences in downstream β-arrestin-dependent signalling events (Nobles et al., 2011).

5. Dimerization

Recent observations that the dimerization state of the receptor can differentially modulate ligand activity have provided a glimpse of a mechanism that could substantially increase the possibilities for ligand functional selectivity at GPCRs. It has long been established that GPCRs can self-associate to form homodimers, interact with other GPCRs as heterodimers and form higher-order oligomers; however, the effect of dimerization on the signalling properties of a receptor are not so clear (Maurice, Kamal, & Jockers, 2011). Recently, Urizar et al. (2011) demonstrated that the dimerization state of the D2 dopamine receptor (D2R) changes the pharmacological properties of its ligands. The D2R agonist R-(-)-10,11-dihydroxy-N-n-propylnoraporphine (NPA) has found to have a 10-fold greater potency to stabilize a G_i-coupled conformation when the D2R was heterodimerized with the D1 dopamine receptor compared to the D2R homodimer. Conversely, two other D2R agonists, dopamine and quinpirole, exhibited the same potency for the heterodimer as the homodimer. Similar results were observed for the μ- and δ-opioid receptors, where heterodimerization altered the binding affinity of the ligands for the receptors and changed the coupling preference of the heterodimer from G_i to a pertussis-insensitive G protein (George et al., 2000). In another study, the SNSR-4 somatosensory receptor was observed to exert a dominant negative effect on δ-opioid receptor signalling following heterodimerization, effectively inhibiting its activation by δ-opioid receptor-selective ligands or the bivalent agonist bovine adrenal medulla peptide 22 (BAM22) (Breit, Gagnidze, Devi, Lagacé, & Bouvier, 2006). Altogether these data indicate that ligands may not only exhibit selectivity for effector-specific receptor conformations, but also for different dimerization states of the receptor. Future research will undoubtedly reveal

whether this is a common property of ligand-receptor relationships and the extent of signalling diversity that can be generated through such a mechanism.

6. Selective antagonism

Another interesting but less characterized mode of ligand functional selectivity is the observed selectivity of certain antagonists to differentially inhibit the signalling repertoire of a given receptor. For example, while the type B cholecystokinin receptor (CCK_BR) antagonist L365,260 produces similar inhibitory effects on agonist-promoted IP and AA production, another CCK_BR antagonist, RB213, was found to exhibit a selectivity towards the inhibition of IP production, with an IC_{50} nearly 200x lower than for AA accumulation (Pommier et al., 1999). Similar observations have been made for antagonists of the tachykinin NK1 receptor (Maggi, Patacchini, Meini, & Giuliani, 1993; Sagan, Chassaing, Pradier, & Lavielle, 1996). These data demonstrate that even neutral antagonists, which have no intrinsic efficacy to activate effectors, can still exhibit selectivity for populations of receptors coupled to specific effectors and thus selectively prevent access to the engagement of certain pathways by agonists. This notion that effector coupling can change the conformation of the receptor and, consequently, its affinity for ligands will be expanded upon in *Section 5: Conformational basis of functional selectivity* (page 35).

7. Physiological and clinical relevance

While many of the examples of ligand functional selectivity provided above demonstrate biased signalling among synthetic ligands targeting GPCRs, several receptors are known to have multiple endogenous ligands that collaborate to control receptor activity in normal physiological responses. Indeed, several examples of biased signalling among endogenous ligands have been observed, including those targeting the β_2AR (Reiner, Ambrosio, Hoffmann, & Lohse, 2010), the CC7 chemokine receptor (Kohout et al., 2004) and the MC4 melanocortin receptor (Nickolls, Fleck, Hoare, & Maki, 2005). The endogenous ligands of the MC4 receptor, for example, have been observed to exhibit selectivity for the pathways engaged by the receptor as well as differences in the ability of ligands to induce receptor internalization. While the α and β forms of the endogenous melanocortin stimulating hormone (MSH-α and MSH-β) inhibit the production of cAMP, stimulate Ca^{2+} mobilization

and induce receptor internalization, the MSH-γ peptide selectively modulates the cAMP pathway, with significantly reduced activity towards the others (Nickolls et al., 2005). Although these studies report differences among endogenous ligands to promote receptor-dependent signalling, determining if and how this functional selectivity contributes to the physiological function of the receptor remains an open and interesting question.

In the case of synthetic ligands, however, various modes of ligand functional selectivity have been demonstrated *in vivo*. The AT1AR biased ligand TRV120027, for example, was shown to have significantly distinct effects on rat cardiac performance compared to unbiased ligands (Violin et al., 2010). Whereas both types of ligands were found to decrease mean arterial pressure by antagonizing a G_q-coupled Ca^{2+} mobilization in vascular smooth muscle cells, only TRV120027 was found to increase cardiac stroke volume and cardiomyocyte contractility, a response presumed to occur through agonism of a β-arrestin-dependent response. In another pre-clinical study, Dewire et al. (2013) capitalized on previous evidence that distinct pathways of the μOR provided either the desired analgesic response to therapy or other, undesired adverse effects. In this study, a biased ligand that selectively modulates G protein-dependent signalling, with no efficacy toward β-arrestin recruitment/signalling was found to provide effective analgesia without the gastrointestinal or respiratory side effects associated with morphine at equianalgesic doses. Walters et al. (2009) were also able to separate the physiological effects of β-arrestin-dependent from G protein-dependent signalling at the GPR107 receptor. In this study, activation of β-arrestin-dependent signalling was found to lead to the adverse flushing response that accompanies the therapeutic antilipolytic effect of nicotinic acid treatment, which is mediated by G protein-dependent responses. In yet another elegant study, (González-Maeso et al., 2003) demonstrated that functionally selective ligands for the 5-HT_{2A} receptor exhibit distinct patterns of transcriptional regulation and lead to different behavioural responses in mice, indicating that differences in the acute signalling responses to ligands can be propagated into more persistent changes in the state of the cell.

While the therapeutic potential of drugs has traditionally been determined according to their affinity and efficacy at a given GPCR target, these studies clearly demonstrate an

addition level of selectivity of ligands for higher order cellular responses, highlighting the promise of biased ligands as potential therapies. Indeed, researchers are beginning to identify instances of increased clinical efficacy of certain functionally selective ligands over unbiased ligands. Carvedilol, a beta-blocker ligand with no intrinsic efficacy towards G-protein-dependent signalling but which engages β-arrestin-dependent ERK1/2 activation through β-adrenergic receptors, led to a significant reduction in mortality of chronic heart failure patients compared to those treated with the unbiased beta-blocker metoprolol (Metra, Cas, di Lenarda, & Poole-Wilson, 2004; Wisler et al., 2007). In another recent clinical study, the biased $AT_{1A}R$ ligand TRV120027, which antagonizes G protein-dependent signalling but promotes β-arrestin-dependent signalling, reduced blood pressure in subjects with enhanced renin-angiotensin system activity, demonstrating a key component of effective heart failure therapy (Soergel, Subach, Cowan, Violin, & Lark, 2013). Future clinical studies will determine if the functional selectivity of this compound compared to traditional, unbiased $AT_{1A}R$ antagonists provides greater therapeutic benefit, as has been demonstrated in pre-clinical studies with the drug in canine models (Boerrigter et al., 2011).

5. Conformational basis of functional selectivity

The preponderance of evidence demonstrating the functional selectivity of ligands cannot easily be explained through the stabilization of a single active signalling conformation of the receptor. It is commonly hypothesized that receptors can instead assume multiple, if not a theoretically infinite number of receptor conformations, with differing abilities to engage and activate effectors. In recent years, several technological advancements have permitted investigations into GPCR structure. Using a wide variety of techniques, researchers are beginning to explore exactly how flexible these molecules are, both structurally and functionally, to provide a more complete understanding of the molecular basis of receptor activation and ligand functional selectivity.

1. Spectroscopic studies

Much of the early structural inquiry into GPCR activation relied on spectroscopic experiments, with the use of fluorescent or bioluminescent probes to measure conformational changes (Gether, Lin, & Kobilka, 1995; Hoffmann et al., 2005). Application of these techniques to the study of ligand functional selectivity suggested that ligands with different profiles could stabilize distinct receptor conformations. By measuring spectroscopic changes in a fluorescent probe fused to the third intracellular loop of the β_2AR, it was observed that the receptor proceeds through multiple conformational intermediates in its activation process and that full and partial agonists could stabilize distinct active receptor conformations (Ghanouni et al., 2001a; Swaminath et al., 2004, 2005). Vilardaga et al. (2005) used an intramolecular fluorescence resonance energy transfer (FRET) approach, fusing fluorescent probes to different regions of the α_2AR to demonstrate that agonists and inverse agonists stabilize distinct receptor conformations upon binding. Galés et al. (2005) employed a similar approach, using bioluminescence resonance energy transfer (BRET) instead to examine how differences in receptor conformation affected the nature of its interaction with G proteins. By comparing the interaction between the α_2AR and Gα_{i1} tagged at two distinct regions in the G protein, this study revealed that ligands stabilize distinct conformations of the receptor-G protein complex that correlated with the reported signalling efficacies of the ligands assessed. Similar results were obtained using BRET for the delta opioid receptor (Audet et al. 2008) and the β_1AR (Galandrin et al. 2008). This latter study compared the receptor:G protein complexes stabilized by isoproterenol, bucindolol and propranolol, which act as full agonist, partial agonist and inverse agonist, respectively, for cAMP production, yet all behave as agonists for ERK1/2 activation. These compounds were shown to stabilize distinct conformations of the β_1AR:G_{i1} complex, perhaps as the first demonstration that functionally selective ligands stabilize distinct active receptor states.

One question that often surfaces when attempting to conceptualize the molecular basis of receptor activation in the context of functional selectivity is whether ligand binding induces a conformational change that subsequently determines the receptor's propensity to couple to effectors, or if ligands instead exhibit selectivity for pre-formed receptor-effector complexes. While many of the early spectroscopic approaches used to demonstrate ligand-

specific receptor conformations could not reliably distinguish between these two mechanisms, some more recent studies, however, have begun to assess these competing hypotheses. In one such study, Yao et al. (2009) reconstituted purified β_2AR tagged with a fluorescent monobromobimane tag into high density lipoprotein (HDL) particles. The design of this study allowed receptor activation to be detected in the absence of effectors to assess ligand-induced conformational changes directly. Indeed, the full agonist isoproterenol was able to induce receptor conformational changes in the absence of G proteins. When a nucleotide-free G_s was added after the addition of isoproterenol, additional conformational changes were observed. Alprenolol, a very weak partial agonist, on the other hand, did not induce conformational changes in the receptor alone but could elicit a conformational change when reconstituted with G_s. ICI118,551, an inverse agonist, exhibited no changes in receptor conformation in the absence or presence of G_s. In another recent study, Rahmeh et al. (2012) demonstrated that functionally selective ligands also stabilize distinct conformations of the vasopressin 2 receptor. In this study, the receptor was tagged with fluorescent sensors in two distinct domains, reconstituted in neutral amphipol particles and challenged with G_s- or β-arrestin-biased ligands. Spectroscopic analysis revealed that distinct conformational events correlated with ligand signalling profiles: changes in transmembrane domain 6 (TM6) correlated with G_s activity and rearrangement of TM7 and the intracellular helix 8 was associated with ligand efficacy for β-arrestin recruitment. Altogether, these data suggest that some ligands may in fact be able to induce receptor conformational changes in the absence of effectors, and that this property correlates with the signalling efficacy of the ligand. However, additional information obtained in these and other studies provide evidence in support of the alternative hypothesis that ligands can stabilize pre-formed receptor conformations. For example, in the study performed by Yao et al. (2009), when G_s is added to the β_2AR-HDL particles in the absence of ligand, the receptor assumed an active conformation similar to the agonist-bound form, indicating the ability of cytoplasmic effectors to similarly alter the conformational state of the receptor. This conformational change induced by G_s also increased isoproterenol binding. Similar effects of G proteins on ligand binding had been observed decades ago using traditional radioligand binding assays (Lefkowitz et al., 1976).

A very similar approach was employed Mary et al. (2012) to assess the effect of different effectors on the conformational state of the ghrelin receptor. These researchers found that reconstitution of the receptor with either G_q or β-arrestin induced different spectroscopic changes in the receptor and that these distinct receptor states were differentially susceptible to further conformational changes induced by different classes of ligands. In another study, Azzi et al. (2001) demonstrated that overexpression of the heterotrimeric G_s in Sf9 cells co-infected with the β_2AR decreased the number of binding sites accessible to the inverse agonists ICI118,551, propranolol and cyanopindolol compared to when the receptor was infected alone, to an extent that correlated strongly with the relative efficacies of the ligands. In addition, chronic pre-treatment of HEK293 cells with cholera toxin to downregulate the expression of G_s correspondingly increased the number of binding sites for inverse agonists. Thus, pre-coupling to effectors can induce a conformational change in the receptor that can substantially change its affinity for certain classes of ligands, engendering a selectivity of ligands for distinct receptor populations coupled to different signalling events.

Altogether these data suggest that distinct receptor conformation changes determine both the intrinsic efficacy of a ligand for a pathway (i.e. full, partial or inverse agonist), as well as the coupling of the receptor to different effector pathways. Furthermore, these studies also reveal the bidirectional influence of both ligands and effectors on the conformational state of the receptor: while the conformational changes induced upon ligand binding can influence the coupling of effectors, effectors can also induce conformational changes in the receptor that change its affinity for ligands. Altogether, these studies demonstrate that the conformational "induction" versus conformational "selectivity" hypotheses need not be mutually exclusive mechanisms. In fact, it is quite likely that the molecular basis of functional selectivity involves the contribution of both mechanisms.

2. Crystal structures

Although useful to demonstrate the differences in receptor conformations stabilized by distinct classes of ligands, the spectroscopic studies described above only provide an approximation of the structural determinants that direct ligand functional selectivity. However, the recent elucidation of three-dimensional structures for several GPCRs has revealed key insights to the precise structural determinants of receptor activation by different classes of ligands (Reviewed by Audet & Bouvier, 2012). The long sought after three-dimensional structures of both antagonist-bound (Jaakola et al. 2008; Wu et al. 2010; Zhang et al. 2012; Cherezov et al. 2007; White et al. 2012; Manglik et al. 2012; J. Huang et al. 2013; Kruse et al. 2012; Warne et al. 2008; Chien et al. 2010; Rasmussen et al. 2007) and agonist-bound (Rasmussen et al. 2011; Rosenbaum et al. 2011; Rasmussen et al. 2011; Xu et al. 2011) GPCRs, have provided a much greater understanding of the specific conformational events that mediate the transition between what are considered "inactive" and "active" receptor states, respectively. These studies have elucidated key details about these distinct functional states of the receptor, including the critical contacts made between bound ligands and residues in the binding pocket for agonists and antagonists, the important intramolecular interactions that stabilize each state, and the dynamic structural rearrangements that occur when transitioning from one state to the other.

Transition to the active, agonist-bound state of the receptor involves several important conformational changes in distinct regions of the receptor. First, agonist binding promotes a contracting of the orthosteric binding site of the receptor by approximately 1 Å compared to antagonist-bound structures, as observed in several different receptors, including the β_1AR (Warne et al., 2011), β_2AR (Rasmussen et al. 2011; Rosenbaum et al. 2011; Rasmussen et al. 2011) and the adenosine A2 receptor (Xu et al. 2011). These changes in the binding pocket reveal important differences between the binding modes of antagonists and agonists. In the agonist-bound conformations of the β_2AR, ligands form a hydrogen bond with $Ser^{5.46}$ (superscript refers to Ballesteros‑Weinstein nomenclature; see Ballesteros, 1995), ultimately changing its conformation to break a hydrogen bond with TM3 (Warne et al. 2011; Rasmussen et al. 2011; Rosenbaum et al. 2011; White et al. 2012; Venkatakrishnan et al. 2013). Although the majority of the structure is practically identical to the antagonist-bound inactive conformations that had previously been crystalized, the authors believe that

these agonist-specific changes in the binding pocket are prerequisites to attaining the fully active conformation. Additional structural information about the agonist-induced conformation was obtained upon crystallization of the β_2AR in complex with either the heterotrimeric G_s protein or with a G protein-mimetic nanobody (Rasmussen et al. 2011; Rasmussen et al. 2011). These structures revealed other important changes in the receptor below the binding pocket and at the cytoplasmic interface that are thought to represent a more fully activated receptor (see Figure 2). In addition to the contraction of the binding pocket, these structures also revealed a large outward movement (approximately 14 Å) and bending of TM6 that permits access of the interior of the receptor to the C-terminus of the Gα subunit. This repositioning of TM6 is associated with a change in the conformation of $Ser^{5.46}$ in the binding pocket, positioning it closer to the agonist, potentially revealing a mechanism by which structural changes in the binding pocket are propagated to other regions of the receptor to promote effector engagement. This outward movement of TM6 is accompanied by a breaking of the "ionic lock", a microconformational event thought to play a critical role in the receptor activation process. This ionic lock refers to the double salt bridge formed near the cytoplasmic surface between $Arg^{3.50}$ in the conserved "DRY" motif and the adjacent $Asp^{3.49}$ in TM3 and $Glu^{6.30}$ in TM6 is believed to stabilize the receptor in an inactive state (Ballesteros et al. 2001). The conformational changes associated with the movement of TM6 lead to the neutralization of $Asp^{3.49}$, to destabilize the ionic lock and permit the interaction between $Arg^{3.50}$ and residues in the carboxy tail of the G protein.

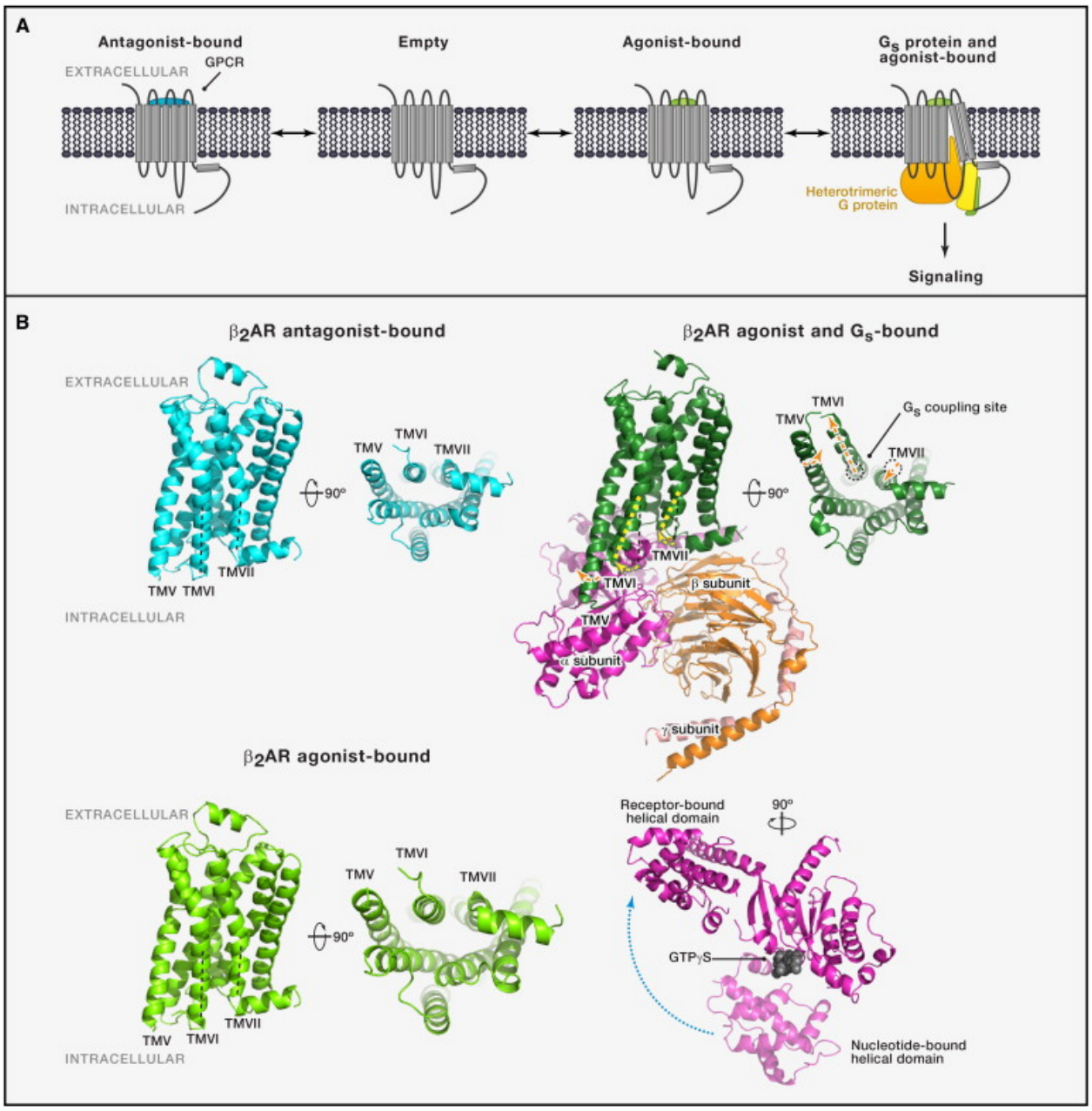


Figure 2: Structural differences in the antagonist- and agonist-bound β_2AR.
(A) Schematic outlining the transition of the receptor to distinct conformations following ligand binding and G protein engagement. (B) Side and cytoplasmic views of β_2AR structures. The large outward movement and bending of TM6 compared to the antagonist-bound state (top-left) is indicated by the orange arrows in the β_2AR agonist- and G_s-bound structure (top-right). It should be noted that these differences are not observed in the β_2AR agonist-bound state (bottom-left). The structural rearrangements in G_s induced upon interaction with the β_2AR are also illustrated (bottom-right). Adapted from Audet and Bouvier (2012)

Researchers have begun to use these structural biology techniques to explore the ability of different signalling classes of ligand to stabilize distinct receptor conformations. Warne et al. (2011) obtained structures of a thermostabilized turkey β_1AR complexed with several full and partial agonists. Although all of these structures ultimately resembled antagonist-bound, inactive conformations (due in part to the thermostabilizing mutations that preferentially bias the receptor towards the inactive conformation; see Serrano-Vega et al., 2008), key differences in the binding pocket were found between full and partial agonists. Although both sets of ligands form hydrogen bonds with $Ser^{5.42}$ and induce a contraction of the binding pocket, only the full agonists form the key hydrogen bond with $Ser^{5.46}$ discussed above. In another study, the same group obtained crystal structures of a thermostabilized avian β_1AR bound to the functionally selective ligands bucindolol and carvedilol (Warne, Edwards, Leslie, & Tate, 2012). Although these ligands show only weak partial agonist or inverse agonist activity, respectively, to G protein-dependent pathways, both act as agonists towards β-arrestin-dependent pathways such as the activation of ERK1/2 (Galandrin et al., 2008). Interestingly, neither of these ligands promoted the contraction of the binding pocket characteristic of agonists, yet they both form hydrogen bonds with $Ser^{5.42}$, similar to partial agonists. Bucindolol and carvedilol also make additional contacts between the aromatic substituents at their amine ends and receptor residues towards the extracellular end of the binding pocket in helices 2, 3 and 7 and extracellular loop 2. Otherwise, these ligands stabilize conformations very similar to antagonist-bound structures. While receptor co-crystallization with G proteins or β-arrestin might elucidate further structural distinctions in the conformation stabilized by these functionally selective ligands compared to unbiased agonists, these data still suggest that distinct receptor states promote G protein coupling and β-arrestin recruitment.

In yet another attempt to further elucidate the structural basis of functional selectivity, Wacker et al. (2013) compared the structure of the 5-HT_{2B} receptor bound to the β-arrestin-biased ligand ergotamine (ERG) to the structure of the 5-HT_{1B} receptor, through which ERG shows no biased agonism (Wang et al. 2013). One key conformational region shown to differ between the two structures is the so-called P-I-F motif ($Pro^{5.50}$, $Ile^{3.40}$, and $Phe^{6.44}$) at the base of the ligand pocket. For the 5-HT_{1B} receptor at which ERG is unbiased,

this motif very closely resembles that of the active state β_2AR-G_s structure (Rasmussen et al. 2011). However, in the 5-HT_{2B} receptor this region adopts an "intermediate active" state, with the Pro and Ile resembling the active structure and the Phe adopting a conformation more similar to the inactive β_2AR structure. In addition, ERG promotes a more active-like conformation of TM6 in the 5-HT_{1B} receptor, which is believed to be one of the main structural determinants of G protein activation. The conformation of TM7, which has been implicated in the activation of β-arresting signalling (Rahmeh et al., 2012), was found to be very similar to the active β_2AR structure for both 5-HT receptors bound to ERG. The authors found additional differences in the extracellular end of TM5, which in the 5-HT_{2B} conformationally constrains the receptor, potentially preventing the rearrangements of TM6 typical of a fully activated receptor.

Altogether these data provide strong evidence that ligand functional selectivity is due, at least in part, to distinct structural rearrangements in the receptor that likely alter its ability to couple to and engage effectors.

3. Nuclear magnetic resonance studies

As informative as the crystal structures have been in understanding the structural basis of receptor activation and ligand functional selectivity, several key aspects cannot be addressed due to the inherent limitations of the method. First, it remains unclear whether functionally distinct ligands stabilize single, distinct receptor conformations with different affinities for effectors or if ligands instead can select from multiple possible active states. Early spectroscopic studies revealed that, indeed, receptors exist in two distinct states following agonist treatment (Ghanouni et al. 2001). However, since in the crystallization process usually only the most stable structure forms a crystal, it is very difficult to obtain structures for such alternative conformations, if they exist. Secondly, crystal structures capture a static representation of receptor structure and thus cannot provide information regarding any conformational dynamics that may be important for function. An alternative structural biology approach that may possess the ability to address some of these aspects is nuclear magnetic resonance (NMR). Similar to the crystallization of GPCRs, only recently

have researchers begun to overcome some of the considerable technical barriers that prevented the use of NMR studies for these large membrane proteins.

In perhaps the first NMR study to examine structural differences in the receptor conformations stabilized by different classes of ligands, Bokoch et al. (2010) assessed changes in the extracellular surface of the β_2AR by selectively labeling lysine residues with [^{13}C]-methyl groups. This approach revealed distinct conformations of the extracellular loops 2 and 3 between an agonist, inverse agonist and neutral antagonist, which the authors hypothesized could change the nature of the binding pocket as well as the cytoplasmic domains that interact with effectors. In a more recent study of the β_2AR using site-specific ^{19}F-NMR, Liu et al. (2012) observed that the cytoplasmic ends of TM6 and TM7 occupy two distinct conformational states, and that the relative population of these two conformations correlated with the signalling profile of the ligand. Full, unbiased agonists, such as isoproterenol, caused large conformational changes in both TM6 and TM7. Partial agonists also induce changes in both TM6 and TM7, with the magnitude of these changes correlating well with the intrinsic efficacy of the ligand. The β-arrestin-biased ligands carvedilol and isoetharine, however, strongly affect only the equilibrium of TM7, with very little effect on TM6. These data clearly demonstrate that the β_2AR can occupy at least two distinct conformations upon ligand binding and that the relative occupancy of these states might explain the signalling profile of the ligand. This group more recently extended this approach, using site-specific [^{13}C] labeling of the ε-methyl groups of methionines in the β_2AR (Nygaard et al., 2013). This labeling of methionines close to the binding pocket (Met$^{2.53}$) and between the binding pocket and the cytoplasmic face of the receptor (Met$^{5.54}$, Met$^{6.34}$ and Met $^{6.41}$) permitted additional insights into receptor activation. First, both when unliganded and when bound to the inverse agonist carazolol, the receptor occupies two different conformations as demonstrated by two distinct peaks in the HSQC spectrum for Met$^{2.53}$. These shifts were shown using molecular dynamics (MD) simulations to correspond to two distinct conformations of TM7, which change considerably the chemical environment around Met$^{2.53}$. Binding of the agonist BI-167107, on the other hand, also stabilizes multiple conformational states; however, these conformational changes are distinct from the unliganded or the carazolol-bound receptor. Furthermore, these agonist-

bound conformations are also distinct from the conformations stabilized by the G protein-mimetic nanobody and when both agonist and nanobody are present. In combination with MD simulations, the authors propose that the receptor transitions between one or more intermediate states following agonist binding and that only when bound by both agonist and G protein (or a G protein-mimetic nanobody) does the receptor reach its fully active state. This conformational heterogeneity may also allow for other G protein or non-G protein effectors to engage these "intermediate" states, providing a plausible structural basis for the coupling of multiple effectors and potentially for ligand functional selectivity if these alternative states are preferentially stabilized by biased ligands.

While the studies above have provided extraordinary structural evidence of distinct receptor signalling states, they do not permit complete structural determination of ligand-bound receptors. Recent developments in solid-state NMR (SSNMR) have begun to provide even greater resolution of the structural determinants of receptor activation and ligand-biased signalling. One additional advantage of SSNMR over crystallography to determine three-dimensional structure is its ability to assess unmodified receptors under physiological conditions. This approach was recently employed by Park et al. (2012) to elucidate the structure of the CXCR1 chemokine receptor in a phospholipid bilayer. While sharing many structural similarities with the related CXCR4, which was previously determined using x-ray crystallography (Wu et al., 2010b), the use of SSNMR allowed the complete, native structure of CXCR1 to be determined, providing key details regarding the structural arrangement of its intra- and extracellular loops. This groundbreaking study should provide the blueprint for further investigations of the mechanisms of receptor activation and ligand-biased signalling by combining this SSNMR approach with biased ligands and/or effectors and applying additional NMR measurements (e.g. chemical shifts, etc.) to ascertain differences in overall and local protein dynamics.

6. Experimental assessment of functional selectivity

Recent development in the experimental approaches to monitor and quantify cellular signalling events have provided extraordinary insight into the functional effects of biased

signalling at GPCRs. A thorough understanding of the extent of functional selectivity and its influence on cell physiology will be crucial to exploit its merits for therapeutic benefit. However, to be most effective in the treatment of a particular disease we must obtain an exhaustive description of the pharmacology of ligands for all of the pathways in the signalling repertoire of a given receptor targeted in a given pathology.

1. Measurement of discrete signalling outcomes

The evaluation of ligand efficacy at a given receptor has typically involved the measurement of a single, discrete signalling event (e.g. production of second messengers, activation of ion channels, etc.). Due to their simplicity, relatively low cost and adaptability to high throughput screening strategies, such surrogate readouts for receptor activation have been used almost universally to identify compounds with a desired pharmacology (i.e. agonist, antagonist or inverse agonist) for a given receptor. This approach has been extremely fruitful in the discovery of drugs to treat a wide variety of human pathologies. However, we now know that receptors can couple to multiple signalling pathways and that the pharmacology of a ligand is dependent on the signalling pathway being measured. As a result, several novel screening approaches have been employed in recent years to take into account possible functional selectivity among compounds at a receptor of interest. These screens often measure multiple signalling endpoints with different and often overlapping endpoints. The development of various *biosensors* that can monitor a diverse array of signalling events has been particular effective to this end. Often combining synthetic biology approaches with BRET or FRET technology, these biosensors allow the real-time monitoring of signalling events such as second messenger production, activation of kinases, protein translocalization and protein:protein interactions in living cells (Breton, Lagacé, & Bouvier, 2010; Charest, Terrillon, & Bouvier, 2005; Depry, Allen, & Zhang, 2010; Fosbrink, Aye-Han, Cheong, Levchenko, & Zhang, 2010; Gorokhovatsky et al., 2004; Harvey et al., 2008; Hodgson, Shen, & Hahn, 2010; Huwiler, Machleidt, Chase, Hanson, & Robers, 2009; Jiang et al., 2007; Nishioka, Frohman, Matsuda, & Kiyokawa, 2010). The development of such biosensors to monitor a vast array of signalling events allows multiple signalling

endpoints to be assessed in homogeneous assay conditions, avoiding potential confounding factors that can arise when different assay formats are used instead to measure different signalling events (see *Section 6.4*, page 54). Furthermore, by designing biosensors with unique emission spectra, several different biosensors can be monitored simultaneously, or *multiplexed*, in the same cell, significantly reducing the time and resources required to screen multiple endpoints for a given target. While biosensors provide a rapid assessment of ligand signalling profiles towards multiple endpoints in a single assay format, validation studies are essential to confirm the results obtained since the overexpression of proteins involved in a given signalling response (e.g. receptors, G proteins, effectors) as biosensors can potentially influence the cellular response to ligand treatment. Label-free techniques to monitor ligand signalling activity, for example, represent an alternative and complementary approach that avoids the potential for experimental artifact associated with the overexpression of signalling proteins (see *Section 7*, page 56)

2. Ligand signalling fingerprints

Screening multiple signalling endpoints at a given receptor target allows for a more comprehensive characterization of the overall signalling activity of a given compound, potentially revealing functionally selective ligands among a compound library. Such a screening strategy can thus reveal ligand signalling fingerprints representative of their overall pharmacology at a receptor, allowing a classification of ligands far beyond the simple agonist vs. antagonist grading. This more *textured* approach to ligand classification provides several key advantages. For one, the precise receptor signalling event underlying a given pathology or its treatment is often unclear. If the signalling event used in a single endpoint screen is unrelated to the therapeutic effect of the receptor, the predictive power of the screen to identify potential drugs could be limited. Furthermore, if additional pathways not assessed in the screen contribute to the clinical response, either therapeutically or in the occurrence of adverse effects, the screen will be blind to these factors. By monitoring multiple endpoints and obtaining ligand signalling fingerprints, one can obtain not only *hits* for the receptor target but *different types* of hits. Distinct functional groups of ligands can then be validated in models of the disease to identify the compound

class with the greatest therapeutic potential, a strategy that could also further elucidate the signalling events underlying a given pathology (Figure 3).

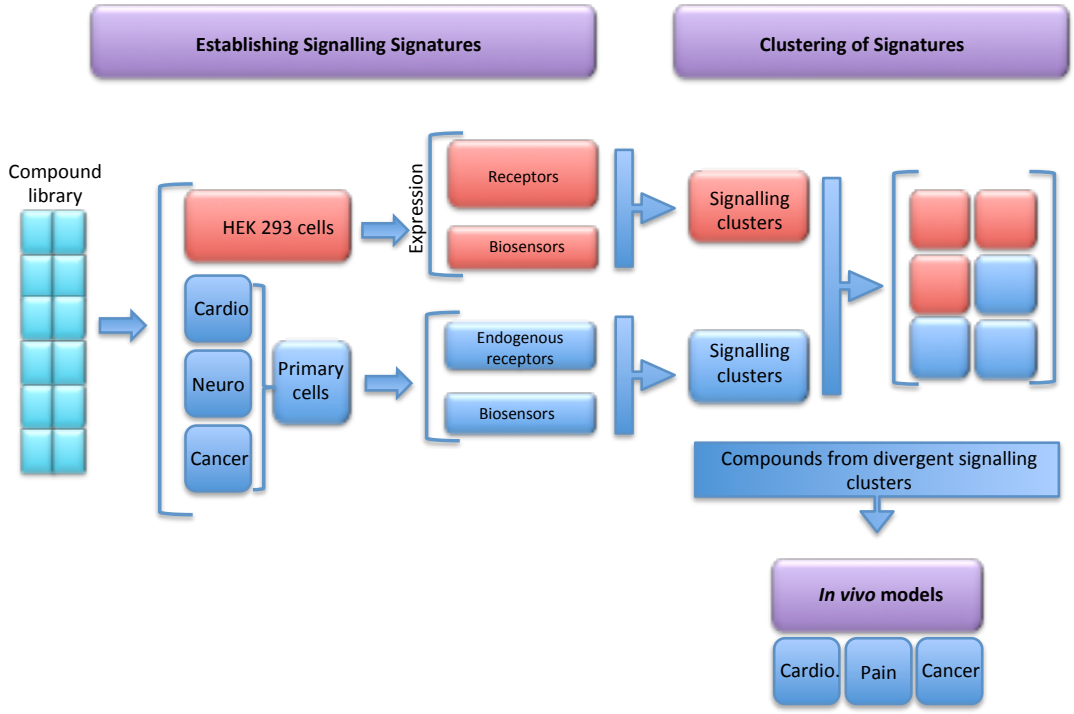


Figure 3: Exploiting functional selectivity for drug discovery.
This schematic details a workflow to assess ligand functional selectivity among a compound library to identify different classes of compounds for further *in vivo* testing. Biosensors measuring distinct signalling endpoints (e.g. cAMP production, kinase activation, G protein coupling, etc.) are expressed heterologously in both an immortalized cell line (e.g. HEK293 cells) in which the receptor of interest would be co-expressed, as well as in primary cells representing the relevant physiological context (e.g. cardiomyocytes, neurons, cancer cell lines, etc.) endogenously expressing the receptor of interest. Compounds would then be clustered according to the overall signalling profiles obtained using the biosensors in each cell line. Individual clusters representing different functional classes could be identified and prototypical members of each cluster could be assessed in *in vivo* models of the disease of interest.

3. Quantifying ligand bias

Screening strategies, which are designed to provide a rapid and simple assessment of the activity of a large quantity of compounds at a receptor, often only provide a rough estimate of the maximal efficacy of a ligand for a signalling event. Secondary screens of the hits obtained are used to further characterize other pharmacological parameters, such the affinity, potency and selectivity for the target. Even screening strategies that assess multiple signalling endpoints only provide an approximation of the overall pharmacology of a given ligand, including any potential biased signalling. However, disparities in the efficacy of ligands towards multiple pathways do not always reflect ligand bias at a receptor. Indeed, differences in the amplification of pathways themselves and/or the sensitivity of the assays used to measure them could artificially create apparent bias. For example, two ligands, A and B, might both appear to be full agonists in Pathway 1 while differing in their efficacy toward Pathway 2, behaving as full and partial agonists, respectively. This would seem to indicate that Ligand B shows a bias toward Pathway 1. However, if the sensitivity of the assay for Pathway 1 is less than Pathway 2 or if it is subject to greater amplification, these differences in efficacy may be artificial. Ligand B could simply be a partial agonist for both pathways, yet its activity is overestimated due to limitations in the upper limit of the assay for Pathway 1 or because the cellular components mediating this pathway are saturated at a lower level of receptor activation.

Several other scenarios do provide strong evidence for ligand bias at a receptor. First, the situation described above cannot account for *reversals* in the efficacy of a ligand towards multiple pathways, from an agonist in one pathway to an inverse agonist in another. In addition, changes in the *rank order* of efficacy or potency among a set of compounds also strongly suggest functional selectivity among the ligands. However, several strategies have recently been proposed to assess biased signalling in more nuanced instances and to provide a truly quantitative description of ligand bias at a given receptor.

The most straightforward approach to visualizing ligand bias is the construction of a *bias plot* (Gregory, Hall, Tobin, Sexton, & Christopoulos, 2010), which plots the amount of signal produced for two pathways in response to equimolar concentrations of a ligand (Figure 4).

If the ligand is unbiased the curve produced should be linear with a slope of 1, since equal concentrations of agonist produce signals of equal strength for the two pathways. If an agonist shows bias towards one of the pathways, it will produce a curve that resides on the diagonal half of the plane that is closest to the axis plotting the pathway to which the agonist is biased. Although simple to construct and interpret, this type of analysis does not differentiate the therapeutically relevant *agonist bias* from the differences in pathway amplification ("system bias") or assay sensitivity ("observational bias") discussed above (Kenakin and Christopoulos 2012). The influence of these other types of biases is often addressed with the inclusion of a reference ligand, which is usually the endogenous ligand for the receptor as it is often assumed to represent a prototypically unbiased agonist. Since this reference ligand is similarly subject to the same system and observational biases, comparing the bias plots for all other ligands relative to this reference ligand allows the separation and visualization of agonist bias among a set of compounds (Figure 4C).

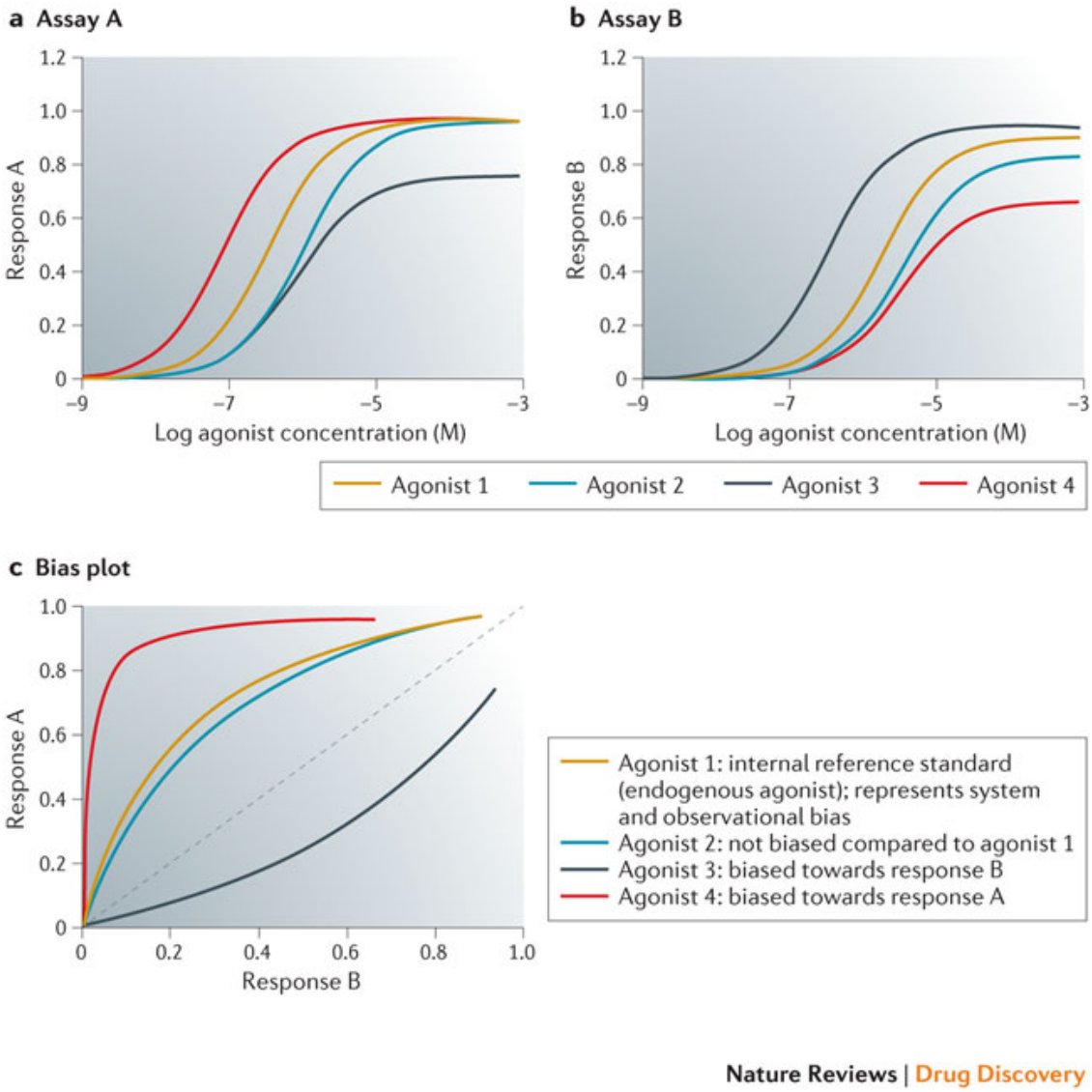


Figure 4: Bias plots to visual ligand functional selectivity.

(a,b) Hypothetical concentration-response curves for four agonists toward two distinct pathways, A and B. Data are normalized to the maximum response of the highest efficacy compound toward each pathway. (c) A bias plot is obtained by plotting the normalized responses of ligands toward each pathway at equimolar concentrations. Using Agonist 1, the endogenous agonist for the receptor, as a reference, we can visualize biased signalling at the receptor towards these two pathways, taking into account the inherent system and observational biases. Agonist 2, which produces a curve similar to Agonist 1 is not biased compared to this reference compound. Agonist 3, which produces a curve residing closer to the axis describing Response B is biased towards this pathway. Agonist 4, on the other hand, produces a curve indicating a bias towards pathway A. Adapted from Kenakin and Christopoulos (2012)

Several other more quantitative methods have also been proposed that use pharmacological parameters derived directly from the operational model (Black & Leff, 1983) to calculate ligand bias (Kenakin and Christopoulos 2012). Key to these approaches is the τ parameter (see Equation 3), which incorporates the effect of agonist efficacy, receptor density and system coupling strength in a signalling response. Using this parameter, a *transduction coefficient* can be calculated for each pathway engaged by a ligand, τ/K_A. The dissociation constant K_A represents the *operational affinity* of a ligand which is a description of the affinity of a ligand for the receptor species that is coupled to a given pathway (e.g. coupled to G proteins, β-arrestin, etc.) and not its affinity for the uncoupled (or bare) receptor or for the entire population of receptors. K_A can be calculated by fitting experimental data in the form of concentration-response curves for a given pathway with the operational model (Equation 2). Expressed as $\log(\tau/K_A)$, this single term represents the intrinsic efficacy and affinity of a ligand at a receptor coupled to a single, specific effector pathway. By taking into account differences in receptor density and system coupling strength, this parameter ultimately represents the *power* of a ligand to elicit a specific cellular response. In addition, the expression of ligand activity towards a particular pathway as a single number allows comparison between multiple pathways using statistical analyses to assess for any biased signalling. To eliminate potential observational and system biases, these values are then normalized to the $\log(\tau/K_A)$ values of a reference compound (again, often the endogenous agonist of the receptor) to obtain $\Delta\log(\tau/K_A)$ values (which effectively transforms the $\log(\tau/K_A)$ of the reference compound to 1). Finally, $\Delta\log(\tau/K_A)$ values for two different pathways can be compared ($\Delta\Delta\log(\tau/K_A)$) to obtain the *bias factor* representing the relative activity of a ligand to activate one pathway versus another. If the ligand exhibits no bias between the pathways the $\Delta\Delta\log(\tau/K_A)$ will be equal to 1 (as will be the case for the reference ligand). However, if bias exists, the $\Delta\Delta\log(\tau/K_A)$ will be more or less than 1, depending on how the $\Delta\log(\tau/K_A)$ ratio is performed (see Figure 5 for an example).

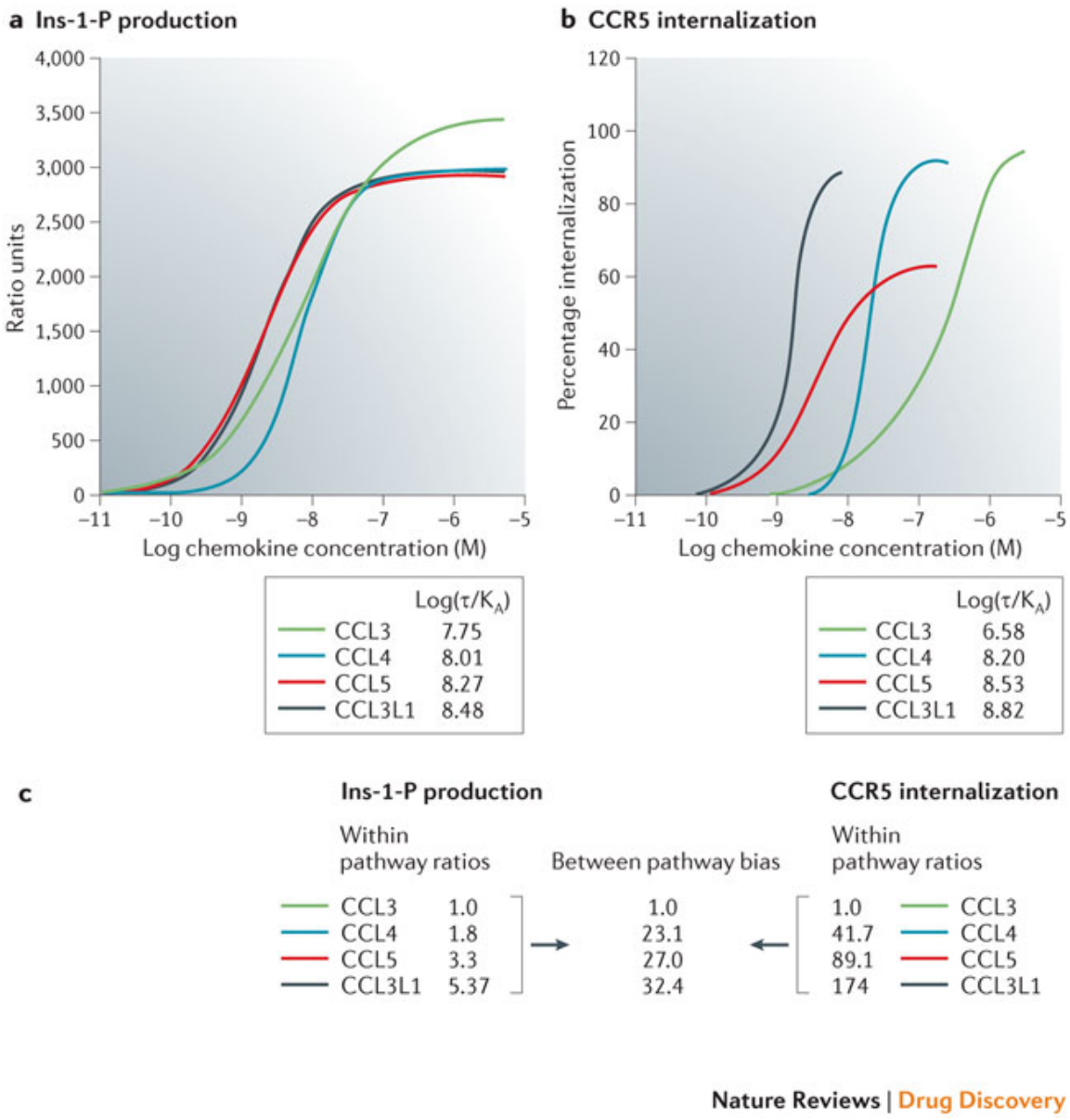


Figure 5: Calculation of bias factors.

(a,b) Concentration-response curves for four CCR5 chemokine receptor agonists toward inositol-1-phosphate (Ins-1-P) production and CCR5 internalization. Log(τ/K_A) values were calculated for each agonist by fitting the concentration-response data with the operational model. (c) Transduction coefficients ($\Delta\log(\tau/K_A)$, "Within pathway ratios") for each pathway were calculated by normalizing $\log(\tau/K_A)$ values for each agonist to the reference compound CCL3. Bias factors ($\Delta\Delta\log(\tau/K_A)$, "Between pathway bias") were calculated as the ratio of transduction coefficients (CCR5 internalization/Ins-1-P production). CCL4, CCL5 and CCL3L1 all show a bias toward CCR5 internalization over Ins-1-P production compared to CCL3, with bias factors greater than 1. Adapted from Kenakin and Christopoulos (2012).

Different, yet related method for calculating bias have also been proposed (Ehlert, 2005; Rajagopal et al., 2011). However, the method described above is probably the most comprehensive and best matches the current theoretical understanding of receptor activation and biased signalling, particularly because it takes into account the changes in receptor affinity that occur upon receptor coupling to different effectors by calculating the operational affinity for each pathway (Kenakin and Christopoulos 2012). Such a method could become indispensible as a follow-up to screening strategies exploiting functional selectivity for therapeutic application and the subsequent medicinal chemistry attempts to enhance the bias of hits by providing a single parameter to evaluate the effects of changes in compound structure on the desired functionality of a compound.

4. Limitations of endpoint assays

Despite continual developments to improve endpoint assays and to quantitatively assess ligand bias from the data obtained, these approaches have several inherent disadvantages that limit their ability to truly reflect the extent of functional selectivity and its influence on the overall cellular response to ligand treatment.

One major limitation of using endpoint assays to characterize the full signalling spectrum of a ligand at a receptor is that the only pathways that can be monitored are the ones that have previously been identified. Although this point might seem obvious, it results in an inevitable systematic bias in screening strategies; the events being monitored are specifically selected and measured explicitly. In this context, it becomes a non-trivial technical challenge to provide an exhaustive description of ligand activity at a given receptor. This issue should not be underestimated considering that the precise cellular determinants of many diseases remain unclear. However, even if most (or all) of the signalling events elicited by a receptor are known, individual assays must be designed, optimized and performed to measure each independently. This can become an extraordinarily time- and resource-consuming process. Additionally, differences in the assay conditions (e.g. live cells vs. lysates, differences in pH, salt concentration, temperature, etc.) or cellular contexts (e.g. different cell lines, receptor/effector expression

levels, adherent or suspended cells, etc.) in which each measurement is made may significantly influence the results obtained.

Even if the issues above are addressed or negligible, the characterization and quantification of biased signalling using single-endpoint-derived data is subject to its own intrinsic limitations. For instance, all of the methods developed thus far only allow the pairwise comparison of two pathways to assess ligand bias. If more than two pathways are assessed, all pairwise comparisons must be made independently and there exists no strategy to integrate these relationships into an *absolute* description of ligand bias. Furthermore, all of these approaches require comparison to a reference compound to establish bias. Since there exists no true measure of absolute bias (which, admittedly, might be conceptually unattainable) one can never be sure that the reference compound used is itself unbiased.

Another potential limitation to using individual endpoint assays to establish ligand bias is that the relationship among pathways and their integration into an overall cellular response cannot be determined. Signalling networks are highly organized to be both responsive and robust to different qualities and quantities of stimuli, consisting of various regulatory and feedback mechanisms that control the cellular response to network modulation. The influence of a given signalling event on the overall cellular response might change depending on the greater signalling context (e.g. the expression or level of activation of other signalling molecules; see DISCUSSION, *Section 3*, page 188). For example, the effect of differences in ligand efficacy towards one pathway could be either diminished or amplified depending on the other signalling events modulated by the ligand. Therefore, the functional significance of bias factors calculated from single endpoint data remains unclear. Additional assays measuring higher-order cellular responses would be necessary to elucidate the physiological and/or therapeutic value of biases observed.

7. The use of cell-based, label-free techniques to assess functional selectivity

Given these limitations in traditional endpoint assays, it would be highly advantageous and complementary to have an assay that can detect the activation of multiple signalling pathways simultaneously and provide an indication of the effect of the integration of these events on the overall cellular response to ligand treatment. To this end, a relatively new approach has been developed that could potentially provide a more "holistic" description of ligand activity. Such cell-based, label-free assays provide an integrative measure of cellular activity by monitoring higher-order cellular responses instead of discrete signalling outcomes.

1. Cellular impedance vs. dynamic mass redistribution

One example of these label-free approaches involves measuring change in cellular impedance following ligand treatment. By measuring changes in the ionic environment at the point of contact between adhered cells and microelectrode sensors spanning the bottom of wells in a microtitre plate ("cell-substrate impedance"), these assays are capable of monitoring any signalling events that converge on changes in cell morphology, adhesion or cytoskeletal dynamics (Yu et al., 2006). Another type of integrative platform relies on measurements of the dynamic mass redistribution (DMR) in cells upon receptor activation. Using an optical biosensor to measure spatiotemporal changes in cellular biomass, these assays monitor the overall reorganization of cellular constituents that accompanies the activation of most signalling pathways (Fang, Li, & Ferrie, 2007). Both techniques provide an integrative readout of cellular activity in real-time, producing complex response curves representing the modulation of signalling networks following ligand treatment. For example, both impedance- and DMR-based platforms have been shown to detect the cellular response to signalling from all four G protein families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) (Scott and Peters 2010). It has been proposed that receptors coupled to each G protein family elicit a characteristic response, producing distinct "signatures" in these assays. However, the cell-type specificity of responses in these integrative systems (Schröder et al., 2010) and the well-recognized ability of many G proteins to promiscuously couple to

multiple G protein subtypes and to engage G protein-independent signalling limits the general applicability of such an assertion.

In fact, it has been established in several studies that the contribution of individual pathways to the overall cellular response can be revealed experimentally through the use of pathway-selective inhibitors (Peters & Scott, 2009; Schröder et al., 2010). The ability of a single assay format to assess multiple signalling events simultaneously, under the same experimental conditions, provides an exciting new experimental platform for the study of ligand functional selectivity.

2. Other applications of label-free assays

Based on their ability to monitor higher-order cellular changes, these techniques have also been useful to study various other aspects of cell biology. Cellular impedance, by monitoring the attachment of cells to microelectrodes, has proved to be well adapted to study factors modulating cell proliferation and death (Al-Ahmadi, Al-Haj, Al-Mohanna, Silverman, & Khabar, 2009; Danussi et al., 2011; Dürr et al., 2012; Ren et al., 2011; Wachter et al., 2012). Using an impedance system to screen for potential anti-mitotic agents, Abassi et al. (2009) were even able to cluster different classes of anti-mitotic compounds by the shape of the resulting impedance profiles as cells went into mitotic arrest. Changes in impedance were found to accurately assess changes in cell adhesion and spreading in response to genetic manipulations or T cell activation (Jarvis et al. 2012; Miller et al. 2012). This platform has also been used to assess viral cytopathogenesis, even distinguishing between viral strains by their resultant cellular responses and assessing the protective effect of human anti-sera (Tian et al., 2012).

3. Advantages of label-free techniques to study GPCR signalling

As discussed above, cellular impedance and DMR-based platforms could represent exciting new tools for the study of ligand functional selectivity, offering several advantages over single endpoint assays. The ability to monitor multiple signalling events simultaneously and dissect the contribution of distinct signalling pathways to the overall response would significant reduce the time and resources required to obtain a comprehensive signalling

profile for a set of ligands. In addition, the ability to assess multiple pathways using a single assay effectively would negate any potential effect of different assay conditions on the results obtained. In particular, the label-free nature of these assays allows signalling events to be measured without altering the endogenous signalling systems. This attribute also permits the study of endogenous receptor activation in their native context or in primary cells isolated from disease models, contexts which are often challenging to study due to their sensitivity to some assay conditions and resistance to genetic manipulation. Thus the influence of biased signalling could be assessed directly in a pathological context. Furthermore, since specific signalling endpoints need not be defined prior to screening, these integrative systems should allow the contribution of pathways not known to be coupled to a given receptor to influence the results obtained, potentially allowing drugs to be classified by their complete signalling profiles at a given receptor.

OBJECTIVES

This thesis will describe the use of cellular impedance measurements to study ligand functional selectivity at GPCRs using the β_2AR as a model receptor system. The β_2AR is an ideal receptor for this purpose as it couples to multiple effectors to activate a number of interconnected pathways (Figure 6). In addition, a large library of compounds exhibiting a wide range of signalling profiles have been identified that target the receptor. This diversity of ligand signalling profiles is highly advantageous as it provides a means to assess the main objective of this thesis: to determine if cellular impedance can be used to differentiate among ligands with distinct signalling profiles at a given receptor.

This thesis will address several specific objectives:

1. Investigate if cellular impedance provides an integrative representation of the multiple signalling events that comprise the β_2AR signalling repertoire (see Article 1, page 63).

2. Determine if ligands with different signalling profiles will generate distinct impedance signatures (see Article 1).

3. Assess the ability of cellular impedance to identify novel signalling pathways for the β_2AR (see Article 1 and Article 2, page 110).

4. Develop a method to cluster compounds into functional classes based on these impedance signatures (see Article 1).

5. Determine if combining pathway-selective inhibitors with impedance profiling provides greater resolution of functional differences among ligands by assessing if and how a given pathway contributes ligand impedance signatures (see Article 3, page 140).

6. Assess the ability of impedance profiling to predict the signalling profiles of an uncharacterized library of β_2AR compounds (see Article 3).

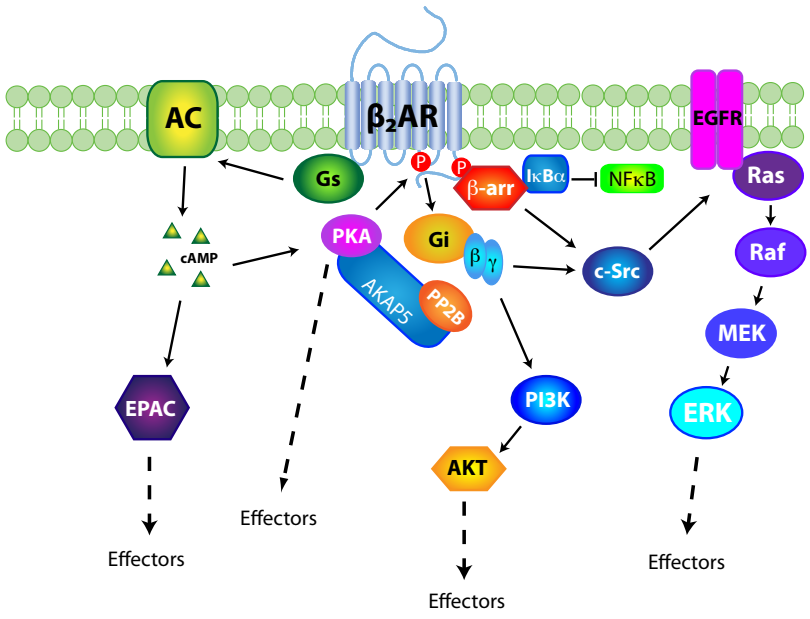


Figure 6: Known components of the β_2AR signalling network.

The β_2AR has been shown to engage a variety of signalling pathways upon activation through the recruitment and/or modulation of multiple G protein and non-G protein effectors. The most characterized of these is the G_s-dependent activation of adenylyl cyclase, leading to the production of cAMP. This increase in cAMP leads to the activation of the cAMP-dependent effectors PKA and exchange nucleotide protein directly activated by cAMP (EPAC), which further propagate the signal to other cellular effectors, leading to a variety of downstream responses. As a mechanism to ensure signalling fidelity and efficacy, β_2AR-mediated cAMP production is spatiotemporally regulated through the actions of the scaffolding proteins AKAP5. This scaffold physically links many of the major components of the cAMP-PKA signalling axis to the β_2AR, including the regulatory subunits of PKA and the protein phosphatase PP2B. Interestingly, the assembly of this complex has been shown to regulate the phosphorylation of the β_2AR by PKA, which leads to a subsequent change in the G protein coupling preference of the receptor from G_s to G_i. This switch in the coupling of G_i to the β_2AR has been shown to contribute to the activation of the ERK1/2 MAPK pathway, in a mechanism whereby the βγ subunits of the G_i protein are proposed to mediate the c-Src-dependent activation of Ras (Daaka et al., 1997). The βγ subunits released from G_i have also been implicated in the activation of the PI_3K/Akt pathway following β_2AR activation (Zhu et al., 2001), which in turn propagates the signal to a number of other downstream effectors. In addition to these G protein-dependent signalling events, several other non-G protein effectors have been found to interact directly with the β_2AR, including β-arrestin1/2. In addition to its role in endocytosis, β-arrestin has been shown to participate in an alternative mechanism linking β_2AR stimulation to ERK1/2 activation in a G protein-independent manner (Shenoy et al. 2006). β-arrestin has also been

observed to regulate NF-κB-dependent transcription by stabilizing a complex between the receptor, β-arrestin and IκB kinase (IκK), preventing the phosphorylation and degradation of IκK and the subsequent activation of NF-κB (Gao et al., 2004).

RESULTS

ARTICLE 1

Impedance responses reveal β_2-adrenergic receptor signaling pluridimensionality and allow classification of ligands with distinct signaling profiles

Wayne Stallaert, Jonas F. Dorn, Emma van der Westhuizen, Martin Audet & Michel Bouvier, 2012. Published in *PLoS ONE* 7(1): e29420. doi:10.1371/journal.pone.0029420

Context/Objectives:

This article represents a proof-of-principle for the use a label-free technique, such as cellular impedance, to characterize and screen for ligand functional selectivity at GPCRs. Using the β_2AR as a model receptor system, we assessed the ability of impedance to functional discriminate among compounds with different signalling profiles at the receptor.

Impedance responses were measured in treated HEK293S cells heterologously expressing the human β_2AR. Treated with the prototypical agonist isoproterenol (ISO) generated a complex impedance curve consisting of multiple distinct features over time. With the use of pathway-selective inhibitors, we assessed the ability of impedance to incorporate the activation of many of the canonical signalling events known to comprise the β_2AR signalling repertoire. This approach revealed both Gs- and Gi-dependent signalling, as well as cAMP and ERK1/2 pathways contributed to the ISO impedance response. We also elucidated the contribution of a novel Ca^{2+} mobilization mechanism to the impedance response generated by β_2AR stimulation. Given the ability of impedance to encode multiple signalling events in a single readout, we then screened a library of functionally selective β_2AR compounds to determine if differences in ligand signalling profiles would be reflected by distinct impedance signatures. A novel computational approach was developed to the quantify differences among the impedance signatures obtained and cluster ligands into distinct compound classes. Compounds were found to cluster into five distinct functional classes, revealing more signalling texture than

previously recognized for the receptor, and correlating with the signalling profiles of ligands towards cAMP production and ERK1/2 activation.

My contribution as first author included the conception and execution of all of the experiments conducted except for the cAMP and ERK1/2 assays. I also participated in much of the analysis, with the exception of the computational approach to cluster impedance signatures. I wrote the first draft of the manuscript and participated greatly in its subsequent revision.

Impedance responses reveal β_2-adrenergic receptor signaling pluridimensionality and allow classification of ligands with distinct signaling profiles

Running title: β_2AR signaling integration and ligand signatures

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Key words: biosensors, drug discovery, G protein-coupled receptors, G protein signaling, MAPK signaling cascade, cellular impedance

ABSTRACT

The discovery that drugs targeting a single G protein-coupled receptor (GPCR) can differentially modulate distinct subsets of the receptor signaling repertoire has created a challenge for drug discovery at these important therapeutic targets. Here, we demonstrate that a single label-free assay based on cellular impedance provides a real-time integration of multiple signaling events activated upon GPCR stimulation. Stimulation of the β_2-adrenergic receptor (β_2AR) in living cells with the prototypical agonist isoproterenol generated a complex, multi-featured impedance response over time. Selective pharmacological inhibition of specific arms of the β_2AR signaling network revealed the differential contribution of G_s-, G_i- and G$\beta\gamma$-dependent signaling events, including activation of the canonical cAMP and ERK1/2 pathways, to specific components of the impedance response. Further dissection revealed the essential role of intracellular Ca^{2+} in the impedance response and led to the discovery of a novel β_2AR-promoted Ca^{2+} mobilization event. Recognizing that impedance responses provide an integrative assessment of ligand activity, we screened a collection of β-adrenergic ligands to determine if differences in the signaling repertoire engaged by compounds would lead to distinct impedance signatures. An unsupervised clustering analysis of the impedance responses revealed the existence of 5 distinct compound classes, revealing a richer signaling texture than previously recognized for this receptor. Taken together, these data indicate that the pluridimensionality of GPCR signaling can be captured using integrative approaches to provide a comprehensive readout of drug activity.

INTRODUCTION

G protein-coupled receptors (GPCRs) are the most abundant class of cell surface receptors, responding to various types of endogenous stimuli including hormones, neurotransmitters and odorants, and are the largest family of therapeutic targets (Imming, Sinning, & Meyer, 2006; Lagerström & Schiöth, 2008). Through interactions with various G protein (Hermans, 2003) and non-G protein effectors (Bockaert, Fagni, Dumuis, & Marin, 2004), GPCRs elicit a diverse array of signaling events, including production of second messengers, activation of phosphorylation cascades, modulation of ion channel activity and transcriptional regulation. Although it was originally assumed that a given GPCR controlled the activity of a single signaling pathway, it is now recognized that individual receptors can elicit multiple signaling events resulting in global changes in cellular physiology, thus highlighting the pluridimensionality of GPCR signaling (Kenakin, 2010). Furthermore, certain GPCR ligands have been shown to differentially modulate distinct subsets of this signaling repertoire, a phenomenon referred to as "ligand-biased signaling" or "functional selectivity" (Stallaert, Christopoulos, and Bouvier 2011; Galandrin, Oligny-Longpré, and Bouvier 2007; Kenakin 2010). A recent review of the published literature indicates that ligand-biased signaling occurs for a large number of GPCRs involved in a diverse array of physiological functions (Kenakin & Miller, 2010). For example, several peptide ligands for the angiotensin AT_{1A} receptor show a clear preference in the activation of β-arrestin-dependent signaling events, yet possess no intrinsic efficacy towards G protein-dependent signaling (Violin et al., 2010; Wei et al., 2003). Even more striking is the inversion of efficacy observed for some β-adrenergic receptor ligands, which act as inverse agonists for cAMP production yet behave as agonists for the activation of the ERK1/2 MAPK pathways (Galandrin and Bouvier 2006; Azzi et al. 2003). Harnessing such ligand functional selectivity could represent a promising avenue in drug development, as the design of compounds that selectively modulate a pathway involved in a given pathology without collateral effects on other pathways could provide therapeutic benefit with a decreased risk of side effects. Indeed, several recent studies have suggested that functionally selective ligands may provide clinically relevant advantages over unbiased ligands at the same receptor (Masri et al., 2008; Noma et al., 2007; Walters et al., 2009).

Yet, detecting the full extent of such ligand "functional selectivity" remains a non-trivial technical challenge and has traditionally involved measuring the relative efficacy of ligands towards distinct pathways engaged by a given receptor using multiple assays. One considerable challenge in obtaining a full description of ligand activity at a given GPCR is the lack of knowledge about the

complete signaling repertoire of most receptors. In addition, monitoring multiple signaling pathways can be time and resource consuming and involves the use of different assay formats with different sensitivity and dynamic ranges that can lead to spurious conclusions.

An integrative approach that could capture the global signaling profile of a ligand in a single assay would therefore greatly facilitate the identification of biased ligands and enable their classification into pharmacologically relevant categories. Besides the potential impact on the drug discovery process, such an approach could also provide greater insight into how the pluridimensionality of GPCR signaling is integrated into an overall cellular response. A string of recent studies has explored the use of such integrative assays to study GPCR pharmacology using label-free, cell-based technologies developed to monitor real-time changes in higher-order cellular events such as morphology, viability, adhesion and mass distribution (Yu et al. 2006; Peters et al. 2010; Peters and Scott 2009; Scandroglio et al. 2010; Schröder et al. 2010). Thus far, however, little effort has been made to understand how the pluridimensionality of GPCR signaling is recapitulated in the responses generated by these assays. One recently developed label-free assay is the measurement of cellular impedance. The use of impedance measurements to monitor global cellular activity is based on the principle that the adhesion of cells directly onto microelectrodes induces changes in the local ionic environment at the electrode/solution interface, conferring an increase in electrode impedance (see Materials and Methods). Any changes in cell morphology and/or adhesion that modulate the physical contact between cell and electrode will be reflected by changes in impedance. Recently, impedance measurements have been used to monitor GPCR signaling (Yu et al. 2006; Peters and Scott 2009; Scandroglio et al. 2010) and to assess general mechanisms of drug action (Abassi et al., 2009).

In the present study, we sought to determine if impedance signatures represent an integrative measure of the receptor signaling repertoire that can be used to differentiate among ligands with distinct signaling profiles. Using the β_2-adrenergic receptor (β_2AR) as a model, we dissected the impedance responses from individual ligand-receptor pairs and associated the activation of specific signaling events with distinct features of the impedance curves. This dissection of the impedance responses led to the identification of a novel β_2AR-mediated Ca^{2+} signaling event, highlighting the strength of such an integrative readout of cellular response to reveal biological processes even at a well-characterized target. Finally, we performed an unsupervised clustering analysis that permitted a rapid and systematic classification of compounds based on the impedance signatures obtained in both heterologous and primary cell cultures, demonstrating that impedance responses represent

signatures that are predictive of the global signaling profiles of β-adrenergic ligands previously shown to display functional selectivity (Wei et al. 2003; Galandrin & Bouvier 2006).

RESULTS

β_2AR activation elicits a multi-featured, time-resolved impedance response.

We first sought to determine if impedance measurements could provide a composite representation of the multiple signaling events elicited upon receptor activation. To assess this possibility, impedance measurements were made in HEK293S cells stably overexpressing the human β_2AR (6HisHA-β_2AR-HEK293S) following treatment with the prototypical β_2AR-selective agonist isoproterenol (ISO). As shown in **Figure 1A**, stimulation of cells with ISO led to a concentration-dependent, time-resolved and feature-rich impedance response. Immediately following ISO-stimulation, the impedance response briefly decreases below zero before quickly reverting to a positive slope, producing what is referred to hereafter as the "transient negative phase" (**Figure 1B**). Subsequently, the impedance rises sharply at first ("rapid ascending phase"), followed by a later "slow ascending phase" before reaching a maximum, after which it decays slowly. This response was found to be β_2AR-specific since the response was blocked by the selective β_2AR antagonist ICI118,551 (ICI) but was not affected by the β_1AR-selective antagonist CGP20712A (**Figure S1A**). In addition, the concentration-response curve describing the maximum impedance response elicited by ISO was progressively right-shifted with increasing concentrations of ICI, indicating a competitive antagonism of the response by β_2AR blockade (**Figure S1B).** Further supporting the β_2AR specificity of the response, we observed a qualitatively similar ICI-sensitive impedance response in the parental HEK293S cell line, which endogenously express low amounts of β_2AR (**Figure S2**). Although the impedance response maintains the same general shape, a decrease in the magnitude of the positive component of the impedance response and a larger transient negative phase was observed.

Further analyses of the impedance responses were performed in the 6HisHA-β_2AR-HEK293S cell line to obtain a more robust response to receptor stimulation. The distinct features of the response (i.e. depth and duration of the transient negative phase, slope of the rapid ascending phase and the maximal impedance response) were differentially modulated by increasing concentrations of ISO, suggesting the contribution of distinct signaling events to discrete components of the impedance response. For instance, concentration-response curves describing either the maximum impedance response (**Figure 1C**) or the slope of the rapid ascending phase (**Figure 1D**), revealed EC_{50} values that differ by more than an order of magnitude. Furthermore, two concentration-dependent components can also be distinguished in the transient negative phase. At low concentrations (i.e.

under 1 μM), ISO promoted a concentration-dependent increase in the magnitude of the minimum (**Figure 1A**). At higher concentrations, however, no further increase in depth was observed, but a quicker recovery from negative impedance values and a shift of the minimum to earlier time points occurred, thus reducing the duration of the transient negative phase. Receptor expression was also found to differentially influence distinct components of the impedance response. As illustrated above, overexpression of the β_2AR selectively increases the positive components of the impedance response while decreasing the duration and amplitude of the transient negative phase (**Figure S2A**). These results further support the above data that the signaling events that contribute to the transient negative phase are fully activated with fewer stimulated receptors. As the stimulus strength (i.e. agonist concentration) or receptor number is increased, the impedance response becomes more positive, likely due to the engagement of another signaling event with a weaker stimulus-coupling. Altogether, these results suggest that the impedance response obtained reflects the integration of multiple signaling events which may be individually represented in the various components of the response.

Impedance responses encode multiple signaling events upon β_2AR activation

β_2AR activation is known to elicit at least two distinct signaling events in HEK293S cells: cAMP accumulation via adenylyl cyclase (AC) stimulation and activation of the ERK1/2 MAPK pathway(Azzi et al., 2003). To directly investigate if these canonical pathways are represented in the impedance response, 6HisHA-β_2AR-HEK293S cells were pre-treated, prior to ISO stimulation, with the AC inhibitor SQ22536 to inhibit cAMP production or the MEK inhibitor U0126 to block ERK1/2 activation. Interestingly, both of these inhibitors led to similar decreases in the slope of the slow ascending phase and the maximum impedance response without affecting the transient negative phase or the rapid ascending phase (**Figure 2A,B**). A similar effect on the impedance response was observed using the K97A dominant negative mutant of MEK1 (**Figure S3**). SQ22536 and U0126 were found to be pathway-specific since they blocked the ISO-stimulated accumulation of cAMP and activation of ERK1/2, respectively, and had no significant effects on the other signaling pathway (**Figure 2C,D**). The observation that cAMP and ERK1/2 pathway inhibition led to similar changes in the impedance response suggested that these pathways may converge on a common mechanism underlying the observed changes in impedance. Consistent with this notion, concurrent inhibition of both pathways did not lead to a greater attenuation of the maximal ISO-stimulated impedance response than inhibition of either the cAMP or ERK1/2 pathway alone (**Figure 2E**).

From the above data, it is clear that cAMP production and MAPK activation are not sufficient to explain the entire ISO impedance response since a considerable residual signal is observed when both of these pathways are inhibited. To further explore the source of this residual response, we examined the role of the G proteins themselves. The β_2AR is known to couple to both G_s and G_i (Daaka, Luttrell, & Lefkowitz, 1997). Chronic treatment with cholera toxin (CTX), which selectively downregulates G_s (Levis & Bourne, 1992), led to a severe attenuation of the impedance response, including a complete loss of the transient negative phase and a significant reduction of the maximum response (**Figure 3A**). Interestingly, CTX inhibited ISO-induced cAMP accumulation to a similar extent as the AC inhibitor SQ22536, with no effect on ERK1/2 activation (**Figure 3B,C**), yet the effect of CTX on the impedance response is clearly distinct from the one observed following inhibition of the cAMP pathway (**Figure S4A**), suggesting the contribution of additional G_s-dependent signaling events other than cAMP accumulation to the impedance response. Selective inhibition of G_i-dependent signaling with pertussis toxin (PTX) led to changes in the impedance response that were different from those caused by CTX (**Fig 3A**). Specifically, a transient negative phase was still observed following PTX treatment, although it was reduced and delayed compared to vehicle treatment. Although both treatments led to significant reductions in the maximum impedance response, the influence of PTX on this phase of the curve was not as severe as CTX. Taken together, these results indicate that G_s and G_i signaling have distinguishable contributions to the overall impedance response. PTX treatment significantly inhibited ERK1/2 activation but did not affect cAMP production (**Figure 3B,C**). However, the contribution of G_i cannot be solely attributed to ERK1/2 activation since complete abolition of this pathway by U0126 (**Fig 2D**) affected the ISO-stimulated impedance response to a lesser extent than PTX (**Figure S4B**), which only partially blocked ERK1/2 activation. Although the differences observed between acute pharmacological inhibition of cAMP production and ERK1/2 activation versus chronic inhibition of Gs and Gi suggest the contribution of alternative G protein-dependent signaling pathways to the impedance response, we cannot exclude the possibility that the chronic treatment with CTX and PTX may affect other downstream components involved in the response.

Next, we assessed the contribution of Gβγ subunits to the impedance response using the pharmacological inhibitor, gallein, which selectively inhibits interactions between Gβγ and effector molecules (Lehmann, Seneviratne, & Smrcka, 2008). Pre-treatment with gallein inhibited ERK1/2 activation, with no effect on cAMP accumulation (**Figure 3B,C)**, and led to an impedance response

with reduced kinetics of the slower ascending phase and a decreased maximum response (**Figure 3D**), The effect of gallein was similar overall to that observed upon inhibition of the ERK1/2 pathway; however, Gβγ inhibition led to an additional loss of the slow decay phase (**Figure S4C**) indicating the existence of additional Gβγ-dependent signaling beyond ERK1/2.

Altogether, these data demonstrate that a variety of G protein-dependent signaling events are integrated in the ISO-stimulated impedance response, including the activation of canonical second messenger pathways (i.e. cAMP and ERK1/2) and additional G protein-dependent events not yet identified.

Clustering analysis of impedance signatures reveals compound classes with distinct signaling profiles

After demonstrating that impedance responses represent the integration of multiple signaling events elicited upon receptor activation, we hypothesized that compounds with different signaling profiles at the β_2AR would generate distinct impedance signatures. Impedance responses were obtained for a series of β-adrenergic ligands (**Figures 4A-I and S5**) at concentrations providing >99% receptor occupancy based on their reported K_d values (Baker, 2005; Hershberger, Wynn, Sundberg, & Bristow, 1990; Hoffmann, Leitz, Oberdorf-Maass, Lohse, & Klotz, 2004). Differences among ligand signatures were determined by comparing the area between individual impedance curves. Visualization of the pairwise differences using a visual assessment of clustering tendency (VAT; see *Materials and Methods*) suggested that the ligands fall into 5 distinct clusters (**Figure S6**), which we confirmed by complete linkage hierarchical clustering (Jain, Murty & Flynn 1999) (**Figure 4J**). With the exception of Group V compounds, which elicit impedance responses of completely negative values, differences between compound classes reflect differences in the specific features defined in our previous analysis of the ISO-promoted impedance response. We see great variation, for example, in the maximum impedance responses generated between compound classes. We also observed clear distinctions between groups in the transient negative phase of the response; Group I compounds elicit a relatively short-lived transient negative phase while Group IV compounds completely lack this feature (**Figure 4F-I**).

To determine the relationship between ligand signaling profiles with the compound classes obtained in the above analysis of impedance signatures, we assayed each of the ligands for their ability to stimulate cAMP accumulation and ERK1/2 activation in conditions identical to those used for the impedance measurements (**Figure 4K,L**). Group I ligands, which include ISO, have high agonist efficacies (full or strong partial agonists) for both cAMP and ERK1/2 responses. Group II

ligands are only weak partial agonists toward the ERK1/2 pathway but elicit as strong cAMP responses as Group I. Group III ligands are weak partial agonists for both cAMP and ERK1/2 pathways. Group IV ligands elicit very weak signaling responses for both pathways. Group V ligands are inverse agonists towards both cAMP and ERK1/2 responses. The compound classes defined by impedance signatures are thus highly predictive of the relative efficacies of ligands to elicit cAMP accumulation and ERK1/2 activation.

The role of intracellular Ca^{2+} in the β_2AR-mediated impedance response

Since neither cAMP accumulation nor ERK1/2 activation contribute to the transient negative phase observed for most ligand impedance signatures, we reasoned that further analysis of this feature could reveal additional texture among ligand signaling profiles. The shape of the transient negative phase can be defined by two parameters, the duration and minimum value of the negative response. Although no significant correlation between these two parameters could be observed when all of the ligands were considered ($r^2 = 0.06$, $p = 0.79$) (**Figure 5**), a strong linear relationship emerged when considering Groups III and IV compounds alone ($r^2 = 0.97$, $p = 9 \times 10^{-5}$), reflecting a fundamental difference in the nature of the transient negative phase between Group I/II ligands and Group III/IV ligands. Indeed, Group I and II ligands have a shorter-lived transient negative phase than would be predicted given their amplitudes (**Figure 5 inset**), suggesting that these compounds may selectively engage a signaling pathway that accelerates the rapid ascending phase thus shortening the duration of the transient negative phase. Given that many GPCRs are capable of activating pathways leading to an increase in intracellular [Ca^{2+}], we assessed whether a β_2AR-mediated Ca^{2+} response could be responsible for accelerating this phase. Consistent with this hypothesis, only Group I and II ligands elicited a significant increase in intracellular [Ca^{2+}], with ISO and epinephrine (Group I) eliciting a much stronger Ca^{2+} response than salbutamol and salmeterol (Group II) **(Figure 6A).** This response was found to be β_2AR-selective since receptor blockade with the β_2AR-selective antagonist ICI completely abolished the ISO-stimulated increase in intracellular [Ca^{2+}] (**Figure S7**). This β_2AR-promoted increase in [Ca^{2+}] was sensitive to the inositol trisphosphate (IP_3) receptor antagonist, 2-aminoethoxydiphenyl borate (2-APB) and the intracellular Ca^{2+} chelator BAPTA-AM (**Figure 6B**), implicating a mobilization from IP_3-gated intracellular stores in the calcium response. Pre-treatment with either 2-APB or BAPTA-AM strongly inhibited the ascending phases of the impedance response (**Figure 6C**), supporting the hypothesis that Ca^{2+} mobilization is a key event in the β_2AR-mediated impedance response.

Interestingly, pre-treatment of cells with either 2-APB or BAPTA-AM significantly modulated the impedance responses not only for ligands in Groups I and II, but also Groups III and IV, which do not elicit a detectable increase in $[Ca^{2+}]$ (**Figure 6A,C-F**). Given that both 2-APB and BAPTA-AM not only inhibit ligand-induced Ca^{2+} mobilization, but also decrease the basal intracellular $[Ca^{2+}]$ (**Figure 6G**), we hypothesized that a minimal concentration of intracellular Ca^{2+} is required to initiate the cellular events underlying the ascending phases of the impedance response. To test this hypothesis, we pre-treated cells with the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (TG), which leads to an increase in intracellular Ca^{2+} by preventing the reuptake of Ca^{2+} thus emptying the intracellular stores. Since the intracellular Ca^{2+} stores are already empty, ISO does not elicit an additional Ca^{2+} release following TG inhibition (**Figure S8A**). Yet, in contrast with what was observed with 2-APB and BAPTA-AM, and despite the absence of a *de novo* Ca^{2+} release, the TG treatment does not inhibit the rapid ascending phase of the impedance response. In fact, it modestly potentiated the maximal response while slightly reducing the slope of the slow ascending phase. Taken together, these data support the notion that it is the intracellular $[Ca^{2+}]$ itself, more than the *de novo* Ca^{2+} release, that is the prime determinant of the ascending phases. The fundamental role of Ca^{2+} in the impedance response is further demonstrated by the effect of the Ca^{2+} ionophore A23187 on the impedance responses. On its own, the ionophore elicits only a weak positive impedance response. However, it greatly potentiates the impedance responses obtained upon stimulation with either β-adrenergic ligands or the direct activator of adenylyl cyclase, forskolin, yielding responses with faster kinetics of the ascending phase and a higher overall maximum response (**Figure 7**). This effect is particularly evident when considering the forskolin-stimulated impedance response, which consists of a long transient negative phase and a slow rise to a relatively modest maximum response in the absence of the Ca^{2+}ionophore. Taken together, these data explicitly demonstrate the importance of Ca^{2+} in the impedance response and indicate that under normal conditions a minimal $[Ca^{2+}]$ is needed to yield an increase in the β_2AR-promoted impedance response while additional Ca^{2+} mobilization by Group I and II ligands further accelerates the rapid ascending phase.

Distinct impedance signatures detected in rat aortic vascular smooth muscle cells

To explore the applicability of impedance-based monitoring of the signaling activity of a GPCR in its native cellular context, we assessed the β_2AR response in rat aortic vascular smooth muscle cells (VSMCs). As was the case in β_2AR-expressing HEK293S cells, ISO induced a response that was completely abolished upon pre-treatment with the β_2AR-selective antagonist ICI (**Figure 8A).**

However, the shape of the impedance response was radically different in VSMCs, indicating that cellular response to receptor activation is cell type-specific. We next assessed the impedance responses induced upon treatment with ligands representing each of the 5 compound classes defined above (**Figure 8B**). Using the same clustering criteria as in the 6HisHA-β_2AR-HEK293S cells, distinct impedance signatures for ISO (Group I), salbutamol (Group II) and labetalol (Group III) were observed. Propranolol (Group IV) and ICI (Group V) generated distinct signatures from these other compounds, but could not be distinguished from each other in VSMCs (**Figure 8C**), emphasizing the cell-type specificity of the response. Altogether, these data indicate that despite the fact that the magnitude and direction of the responses are cell-type specific, quantitative analysis of impedance responses can be used to detect distinct ligand signatures in primary cell cultures.

DISCUSSION

In the present study, we demonstrate that impedance responses provide an integrative assessment of the cellular consequence to GPCR stimulation, representing a holistic readout of the various signaling events elicited in real-time. In addition, clustering analyses of the impedance responses provided a means to sub-classify ligands in a manner that was predictive of their signaling profiles and revealed a richer signaling texture among β-adrenergic ligands than previously envisaged.

Analysis of the β_2AR-promoted impedance response to ISO stimulation revealed the contribution of both G_s and G_i coupling, Gβγ-dependent signaling, as well as cAMP production, ERK1/2 activation and a novel Ca^{2+} mobilization response to the overall changes in cellular impedance. The observation that elements of the impedance response that were blocked upon G_s or G_i inactivation following chronic treatment with CTX and PTX, respectively, could not be fully recapitulated by acute pharmacological inhibition of cAMP or ERK1/2 pathways suggests the contribution of additional G protein-dependent signaling. However, we cannot rule out the possibility that chronic inhibition of G proteins leads to cellular changes (e.g. changes in expression of signaling or adhesion proteins) that indirectly affect the impedance response. Our analysis also provided evidence that both cAMP and ERK1/2 pathways converge on a common downstream effector that mediates their contribution to the impedance response, although the identity of this target remains unknown.

The impedance responses generated following β_2AR stimulation was found to be extremely sensitive to inhibition of intracellular Ca^{2+} mobilization. Although the β_2AR has previously been shown to increase intracellular [Ca^{2+}] through modulation of L-type voltage-gated Ca^{2+} channels (Benitah, Alvarez, & Gómez, 2010) and the ryanodine receptor (Kushnir & Marks, 2010), our data implicate a novel IP_3-gated release of Ca^{2+} from intracellular stores. Ca^{2+} is a well-established regulator of actin cytoskeleton dynamics (Janmey, 1994; Marston, 1995) and plays an important role in controlling cellular adhesion and morphology, through effectors such as cadherins(van Roy & Berx, 2008) and annexins (Gerke, Creutz, &

Moss, 2005), potentially providing a key mechanistic link between β_2AR stimulation and the impedance changes observed. Although we found this *de novo* Ca^{2+} release to contribute to the rapid ascending phase of Groups I and II ligands, the impedance responses of ligands that do not themselves induce a Ca^{2+} mobilization are also sensitive to a disruption of intracellular Ca^{2+} homeostasis, stressing the importance of Ca^{2+} in the cellular mechanisms that initiate an impedance response. We propose that the normal resting concentration of intracellular Ca^{2+} is necessary and sufficient to permit the cytoskeleton to respond to subsequent signaling events, such as cAMP production or ERK1/2 activation, and that ligands that induce an additional increase in intracellular [Ca^{2+}] further sensitize the cell to these signaling inputs leading to an enhancement of the impedance response. Thus, the overall impedance signature of a given ligand truly represents the integration of the signaling pathways activated.

The contribution of multiple signaling events to the overall impedance response challenges some of the efforts that have been made to reduce such integrated responses to the engagement of a single G protein (Peters et al. 2010; Peters and Scott 2009). It is now widely accepted that many GPCRs promiscuously couple to and activate signaling through multiple G protein subtypes (Hermans, 2003) as well as G protein-independent pathways. In the current study, we observed that both G_s- and G_i-dependent signaling were integrated in the overall β_2AR-promoted response. Moreover, the fact that the signatures obtained in VSMCs were dramatically different than those observed in 6HisHA-β_2AR-HEK293S cells highlights the cell-type dependency of the impedance response, which likely reflects differences in the relative abundance of signaling molecules and/or mediators underlying the changes in cell shape and adhesion that give rise to the impedance responses. These observations highlight the inherent complexity of signaling systems and their measurement and demonstrate the diversity of cellular responses that can be generated by the stimulation of a given GPCR in different cell contexts. The ability to detect and dissect these complex cellular responses, as demonstrated in the present study, provides information concerning the full signaling repertoire of a receptor that can be more useful in drug discovery than the attempt to ascribe a single signaling pathway to a receptor.

The fact that multiple signaling events are integrated in the impedance response makes it an appealing platform to classify compounds based on their signaling repertoire. Clustering of the impedance responses generated by a series of β-adrenergic ligands revealed an unprecedented level of ligand texture at the β_2AR, demonstrating the existence of at least five groups of compounds with distinct impedance signatures. Previous work assessing the signaling profiles of β-adrenergic ligands towards cAMP and ERK1/2 pathways alone using endpoint-specific assays proposed the existence of 3 distinct compound classes (Galandrin and Bouvier 2006). The ability of impedance measurements to detect additional ligand diversity likely reflects the contribution of other pathways not previously assessed. Furthermore, differences in ligand signaling profiles between this study and the former demonstrate the critical influence of assay conditions on the reported signaling properties of a ligand. In the present study, cAMP accumulation and ERK1/2 activation were measured in conditions identical to those used for impedance measurements. Without serum starvation, ligands such as propranolol and ICI did not generate a detectable ERK1/2 response, as previously reported(Galandrin and Bouvier 2006; Azzi et al. 2003). However, differences in their respective impedance responses indicated distinctions in the overall cellular response induced by ICI and propranolol, which had not previously been observed.

Although cell-based, integrative assays have begun to be employed in some screening applications (Atienza, Yu, Wang, Xu, & Abassi, 2006; Dodgson, Gedge, Murray, & Coldwell, 2009; Fang & Ferrie, 2008; McGuinness et al., 2009), our results represent a first proof-of-principle of how they can be used not only to identify potential ligands for a given target, but also to differentiate classes of compounds with specific signaling profiles that may correlate with therapeutic or adverse effects. We also demonstrate the unique ability of integrative assays such as impedance measurements to illuminate novel biology not yet revealed by traditional single endpoint assays. The approaches described should be universally applicable to all label-free platforms that provide an integrative cell response, including both impedance-based systems and those based on dynamic mass redistribution. Most certainly, analytical integrative methodologies such as the one presented here, will

provide a more in-depth exploration of cellular signaling dynamics and should be transformative in the future of drug discovery.

MATERIALS AND METHODS

Materials and reagents

Gallein was obtained from Tocris, U0126 from Cayman, and bucindolol was a generous gift from Dr. Michael Bristow (University of Colorado Health Sciences Center, Denver, CO). Cell culture reagents were from Wisent Incorporated. Other reagents were obtained from Sigma-Aldrich unless stated otherwise.

Stable cell lines and transfection

The pIREShygro_6HisHA-β_2AR construct was generated as follows: The 6HisHA-β_2AR gene was amplified by PCR from the previously described vector pCDNA3RSV-6HisHA-β_2AR (Lavoie et al., 2002) using sense and antisense primers containing *BsrGI* and *NheI* restriction sites, respectively. The amplified 6HisHA-β_2AR sequence was digested with *BsrGI/NheI* and then subcloned in a *BsrGI/NheI*-digested pIREShygro3 vector (Clontech, Mountain View, CA). The 6HisHA-β_2AR-HEK392S polyclonal cell line was generated by transfecting the pIREShygro_6HisHA-β_2AR construct in HEK293S cells (Reeves, Thurmond, & Khorana, 1996) and selecting vector-inserted cells by growth in culture media containing 100 µg/ml hygromycin B (WISENT inc, St-Bruno, Quebec, CAN). The 6HisHA-β_2AR-HEK293S stable cell line was found to express 1.07 ± 0.14 pmol of 6HisHA-β_2AR per mg of membrane protein as determined by a ^{125}I-CYP radioligand binding assay using. Cells were routinely grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. For Ca^{2+} assays and impedance assays in the presence of the MEK dominant negative mutant, 6HisHA-β_2AR HEK293S cells were transiently transfected with the obelin biosensor (Markova et al., 2002) or the MEK1-K97A-Flag mutant (Scott et al., 2006), respectively, using polyethylenimine and assayed 48 hours later.

Cellular impedance assay

The xCELLigence system (Roche Applied Science) was employed to measure changes in cellular impedance following ligand stimulation (Solly, Wang, Xu, Strulovici, & Zheng, 2004). Briefly, this assay is based on the principle that the adhesion of cultured cells directly onto an array of equally-distributed microelectrodes embedded at the bottom of

wells of a microtitre plate induces changes in the local ionic environment at the electrode/solution interface, conferring an increase in electrode impedance. As a result, any changes in cell physiological properties that modulate the physical contact between cell and electrode, such as changes in morphology and/or adhesion, will be reflected by changes in the measured impedance, as defined by the cell index variable. A measurement is made in the presence of growth medium prior to cell seeding to determine the background cell index in each well of a 96-well E-plate (Roche Applied Science), which is subtracted from the cell index values generated following cell attachment. Cells were plated at a cell density of 20 000 cells/well and grown for 16-20 hours before ligand addition in the RTCA SP device station (Roche Applied Science). When cells were treated with cholera toxin or pertussis toxin, they were plated at a cell density of 10 000 cells/well, treated with toxins after 24 hours and stimulated with ligand 40 hours after seeding. Experimental variation in cell index values at the time of treatment was limited to ±20%, which considerably increased the reproducibility of the data obtained. Cells were treated with compounds as indicated and cell index values were obtained immediately following ligand stimulation every 20 seconds for a total time of at least 100 minutes. Cell index values were normalized by dividing by the cell index at the time of ligand addition and baseline-corrected by subtracting the cell index obtained in vehicle-treated conditions (see **Figure S9**). All data presented in figures represent means of at least three independent experiments.

cAMP accumulation assay

Cells were plated and grown in 96-well plates and treated with ligand as described for the cellular impedance assay. cAMP accumulation was measured using the HTFR-cAMP dynamic kit (Cisbio). After 10 minutes of ligand stimulation, cells were lysed on ice in the lysis buffer provided by the manufacturer and immediately snap-froze at -80°C for at least 4 hours. After thawing on ice, 10 µl per well of lysate was transferred to a 384-well plate (Lumitrac 200, Greiner Bio-one), and cAMP measurements were performed as per manufacturer's instructions.

ERK1/2 activation assay

Cells were plated and grown in 96-well plates and treated with ligand as described for the cellular impedance assay. ERK1/2 activation was measured using the ERK1/2 AlphaScreen Surefire kit (Perkin Elmer). Cells were lysed on ice after 2 minutes of ligand stimulation in the lysis buffer provided by the manufacturer and placed immediately at -20°C overnight. After thawing on ice, 5 µl were transferred to 384-well ProxiPlates (Perkin Elmer) and ERK1/2 activation was measured as per manufacturer's instructions.

Obelin Ca^{2+} biosensor assay

Cells were plated and grown in 96-well microtitre plates and transfected as described above. Forty-eight hours after transfection, growth medium was replaced with Tyrode's solution (1 mM $CaCl_2$, 140 mM NaCl, 2.7 mM KCl, 900 µM $MgCl_2$, 37 µM NaH_2PO_4, 5.6 mM d-glucose, 8.3 mM $NaHCO_3$ and 12.6 mM Hepes, pH 7.5) and cells were incubated with 5 µM coelanterazine H (Nanolight Technology, Pinetop, AZ, USA) for 2 hours. Intracellular Ca^{2+} concentration is reflected by the magnitude of bioluminescence emitted by the obelin biosensor and data are expressed as relative luminescence units (RLU). Ligands were injected as indicated and bioluminescence was measured for 1 minute per condition using the SpectraMax L Microplate reader (Molecular Devices). Baseline readings were obtained for 5 seconds prior to ligand addition. Peak Ca^{2+} responses represent the maximum response in the largest peak obtained following ligand addition. Peaks were defined to have a minimum width of ten adjacent points.

Data analysis

Data analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA), GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) or MATLAB (The MathWorks, Natick, MA, USA). To quantify the slope of the rapid ascending phase, a line was drawn from two data points after the minimum (to avoid artifacts from shallow minima) to maximum response, and the impedance response was bisected at the point farthest from that line. Then, a second line was drawn from two data points after minimum response to the first bisection point, and the impedance response was bisected again. Slope was calculated as

the ratio between the difference in impedance and the difference in time between two data points after the minimum response and the second bisection point.

Differences in ligand impedance signatures were determined by calculating the area between their impedance curves. The area *A* between two curves *i* and *j* was estimated as

$$A_{i,j} = \sqrt{\sum_{t} (I_{i,t} - I_{j,t})^2}$$

where $I_{i,t}$ is the impedance response of curve *i* at time *t*. Clustering of the data was assessed using the visual assessment of tendency method (Bezdek & Hathaway, 2002). Briefly, arrays of the pairwise dissimilarities were sorted by placing the two ligands with the highest pairwise dissimilarity in the first and last rows, respectively. The array was then populated with the remaining ligands by minimizing the dissimilarity among adjacent compounds and maintaining the same order of compounds in rows and columns. To emphasize cluster structure among similar data points, the square root of the sorted dissimilarity maps were displayed. For complete linkage hierarchical clustering, clustering trees were cut at 0.8 times the median of all pairwise dissimilarities.

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REFERENCES

Abassi, Y. A., Xi, B., Zhang, W., Ye, P., Kirstein, S. L., Gaylord, M. R., … Xu, X. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. *Chemistry & biology*, *16*(7), 712–23. doi:10.1016/j.chembiol.2009.05.011

Atienza, J. M., Yu, N., Wang, X., Xu, X., & Abassi, Y. (2006). Label-free and real-time cell-based kinase assay for screening selective and potent receptor tyrosine kinase inhibitors using microelectronic sensor array. *Journal of biomolecular screening : the official journal of the Society for Biomolecular Screening*, *11*(6), 634–43. doi:10.1177/1087057106289334

Azzi, M., Charest, P. G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., & Piñeyro, G. (2003). Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(20), 11406–11. doi:10.1073/pnas.1936664100

Baker, J. G. (2005). The selectivity of beta-adrenoceptor antagonists at the human beta1, beta2 and beta3 adrenoceptors. *British journal of pharmacology*, *144*(3), 317–22. doi:10.1038/sj.bjp.0706048

Benitah, J.-P., Alvarez, J. L., & Gómez, A. M. (2010). L-type Ca(2+) current in ventricular cardiomyocytes. *Journal of molecular and cellular cardiology*, *48*(1), 26–36. doi:10.1016/j.yjmcc.2009.07.026

Bezdek, J. C., & Hathaway, R. J. (2002). VAT: A tool for visual assessment of (cluster) tendency. In *Proceedings of the international joint conference on neural networks* (pp. 2225–2230).

Bockaert, J., Fagni, L., Dumuis, A., & Marin, P. (2004). GPCR interacting proteins (GIP). *Pharmacology & therapeutics*, *103*(3), 203–21. doi:10.1016/j.pharmthera.2004.06.004

Daaka, Y., Luttrell, L. M., & Lefkowitz, R. J. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature*, *390*(6655), 88–91. doi:10.1038/36362

Dodgson, K., Gedge, L., Murray, D. C., & Coldwell, M. (2009). A 100K well screen for a muscarinic receptor using the Epic label-free system--a reflection on the benefits of the label-free approach to screening seven-transmembrane receptors. *Journal of receptor and signal transduction research*, *29*(3-4), 163–72. doi:10.1080/10799890903079844

Fang, Y., & Ferrie, A. M. (2008). Label-free optical biosensor for ligand-directed functional selectivity acting on beta(2) adrenoceptor in living cells. *FEBS letters*, *582*(5), 558–64. doi:10.1016/j.febslet.2008.01.021

Galandrin, S, & Bouvier, M. (2006). Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol*, *70*(5), 1575–1584. doi:mol.106.026716 [pii] 10.1124/mol.106.026716

Galandrin, Ségolène, Oligny-Longpré, G., & Bouvier, M. (2007). The evasive nature of drug efficacy: implications for drug discovery. *Trends in pharmacological sciences*, *28*(8), 423–30. doi:10.1016/j.tips.2007.06.005

Gerke, V., Creutz, C. E., & Moss, S. E. (2005). Annexins: linking Ca2+ signalling to membrane dynamics. *Nature reviews. Molecular cell biology*, *6*(6), 449–61. doi:10.1038/nrm1661

Hermans, E. (2003). Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacology & Therapeutics*, *99*(1), 25–44. doi:10.1016/S0163-7258(03)00051-2

Hershberger, R. E., Wynn, J. R., Sundberg, L., & Bristow, M. R. (1990). Mechanism of action of bucindolol in human ventricular myocardium. *Journal of cardiovascular pharmacology*, *15*(6), 959–67.

Hoffmann, C., Leitz, M. R., Oberdorf-Maass, S., Lohse, M. J., & Klotz, K.-N. (2004). Comparative pharmacology of human beta-adrenergic receptor subtypes--characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's archives of pharmacology*, *369*(2), 151–9. doi:10.1007/s00210-003-0860-y

Imming, P., Sinning, C., & Meyer, A. (2006). Drugs, their targets and the nature and number of drug targets. *Nature reviews. Drug discovery*, *5*(10), 821–34. doi:10.1038/nrd2132

Jain, A.K., Murty M.N., Flynn, P. J. (1999). Data clustering: a review. *ACM Computing Surveys*, *31*(3), 264–323. doi:10.1145/331499.331504

Janmey, P. (1994). Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annual Review of Physiology*, *56*(52), 169–191. doi:10.1146/annurev.ph.56.030194.001125

Kenakin, T. (2010). Functional Selectivity and Biased Receptor Signaling. *The Journal of pharmacology and experimental therapeutics*. doi:10.1124/jpet.110.173948

Kenakin, T., & Miller, L. J. (2010). Seven Transmembrane Receptors as Shapeshifting Proteins : The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. *Pharmacological Reviews*, *62*(2), 265–304. doi:10.1124/pr.108.000992.265

Kushnir, A., & Marks, A. R. (2010). The ryanodine receptor in cardiac physiology and disease. *Adv. Pharmacol.*, *59*, 1–30. doi:10.1016/S1054-3589(10)59001-X

Lagerström, M. C., & Schiöth, H. B. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nature reviews. Drug discovery*, *7*(4), 339–57. doi:10.1038/nrd2518

Lavoie, C., Mercier, J.-F., Salahpour, A., Umapathy, D., Breit, A., Villeneuve, L.-R., … Hébert, T. E. (2002). Beta 1/beta 2-adrenergic receptor heterodimerization regulates beta 2-adrenergic receptor internalization and ERK signaling efficacy. *The Journal of biological chemistry*, *277*(38), 35402–10. doi:10.1074/jbc.M204163200

Lehmann, D. M., Seneviratne, A. M. P. B., & Smrcka, A. V. (2008). Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation. *Molecular pharmacology*, *73*(2), 410–8. doi:10.1124/mol.107.041780

Levis, M. J., & Bourne, H. R. (1992). Activation of the alpha subunit of Gs in intact cells alters its abundance, rate of degradation, and membrane avidity. *The Journal of cell biology*, *119*(5), 1297–307.

Markova, S. V, Vysotski, E. S., Blinks, J. R., Burakova, L. P., Wang, B.-C., & Lee, J. (2002). Obelin from the bioluminescent marine hydroid Obelia geniculata: cloning, expression, and comparison of some properties with those of other Ca2+-regulated photoproteins. *Biochemistry*, *41*(7), 2227–36.

Marston, S. (1995). Ca2+-dependent Protein Switches in Actomyosin Based Contractile Systems. *International Journal of Biochemistry & Cellular Biology*, *27*, 97–108.

Masri, B., Salahpour, A., Didriksen, M., Ghisi, V., Beaulieu, J.-M., Gainetdinov, R. R., & Caron, M. G. (2008). Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(36), 13656–61. doi:10.1073/pnas.0803522105

McGuinness, R. P., Proctor, J. M., Gallant, D. L., van Staden, C. J., Ly, J. T., Tang, F. L., & Lee, P. H. (2009). Enhanced selectivity screening of GPCR ligands using a label-free cell based assay technology. *Combinatorial chemistry & high throughput screening*, *12*(8), 812–23.

Noma, T., Lemaire, A., Prasad, S. V. N., Barki-Harrington, L., Tilley, D. G., Chen, J., … Lefkowitz, R. J. (2007). beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *Journal of Clinical Investigation*, *117*(9), 2445. doi:10.1172/JCI31901.extracellular

Peters, M F, Vaillancourt, F., Heroux, M., Valiquette, M., & Scott, C. W. (2010). Comparing label-free biosensors for pharmacological screening with cell-based functional assays. *Assay Drug Dev Technol*, *8*(2), 219–227. doi:10.1089/adt.2009.0232

Peters, Matthew F, & Scott, C. W. (2009). Evaluating Cellular Impedance Assays for Detection of GPCR Pleiotropic Signaling and Functional Selectivity. *Journal of Biomolecular Screening*, *14*(3), 246–255. doi:10.1177/1087057108330115

Reeves, P. J., Thurmond, R. L., & Khorana, H. G. (1996). Structure and function in rhodopsin: high level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(21), 11487–92.

Scandroglio, P., Brusa, R., Lozza, G., Mancini, I., Petro, R., Reggiani, A., & Beltramo, M. (2010). Evaluation of Cannabinoid Receptor 2 and Metabotropic Glutamate Receptor 1 Functional Responses Using a Cell Impedance-Based Technology. *J Biomol Screen*. doi:1087057110375615 [pii] 10.1177/1087057110375615

Schröder, R., Janssen, N., Schmidt, J., Kebig, A., Merten, N., Hennen, S., … Kostenis, E. (2010). Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. *Nature biotechnology*, *28*(9), 943–949. doi:10.1038/nbt.1671

Scott, M. G. H., Pierotti, V., Storez, H., Lindberg, E., Thuret, A., Muntaner, O., … Marullo, S. (2006). Cooperative regulation of extracellular signal-regulated kinase activation and cell shape change by filamin A and beta-arrestins. *Molecular and cellular biology*, *26*(9), 3432–45. doi:10.1128/MCB.26.9.3432-3445.2006

Solly, K., Wang, X., Xu, X., Strulovici, B., & Zheng, W. (2004). Application of real-time cell electronic sensing (RT-CES) technology to cell-based assays. *Assay and drug development technologies*, *2*(4), 363–72. doi:10.1089/1540658041850544

Stallaert, W., Christopoulos, A., & Bouvier, M. (2011). Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors. *Expert Opinion on Drug Discovery*, *6*(8), 811–825. doi:10.1517/17460441.2011.586691

Van Roy, F., & Berx, G. (2008). The cell-cell adhesion molecule E-cadherin. *Cellular and molecular life sciences : CMLS*, *65*(23), 3756–88. doi:10.1007/s00018-008-8281-1

Violin, J. D., Dewire, S. M., Yamashita, D., Rominger, D. H., Nguyen, L., Schiller, K., … Lark, M. W. (2010). Selectively Engaging beta-Arrestins at the Angiotensin II Type 1 Receptor Reduces Blood Pressure and

Increases Cardiac. *Journal of Pharmacology and Experimental Therapeutics*, *335*(3), 572–579. doi:10.1124/jpet.110.173005.

Walters, R. W., Shukla, A. K., Kovacs, J. J., Violin, J. D., Dewire, S. M., Lam, C. M., … Lefkowitz, R. J. (2009). Beta-Arrestin1 mediates nicotinic acid–induced flushing, but not its antilipolytic effect, in mice. *The Journal of Clinial Investigation*, *119*(5), 1312–1321. doi:10.1172/JCI36806.1312

Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., & Lefkowitz, R. J. (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(19), 10782–7. doi:10.1073/pnas.1834556100

Yu, N., Atienza, J. M., Bernard, J., Blanc, S., Zhu, J., Wang, X., … Abassi, Y. a. (2006). Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Analytical chemistry*, *78*(1), 35–43. doi:10.1021/ac051695v

FIGURES

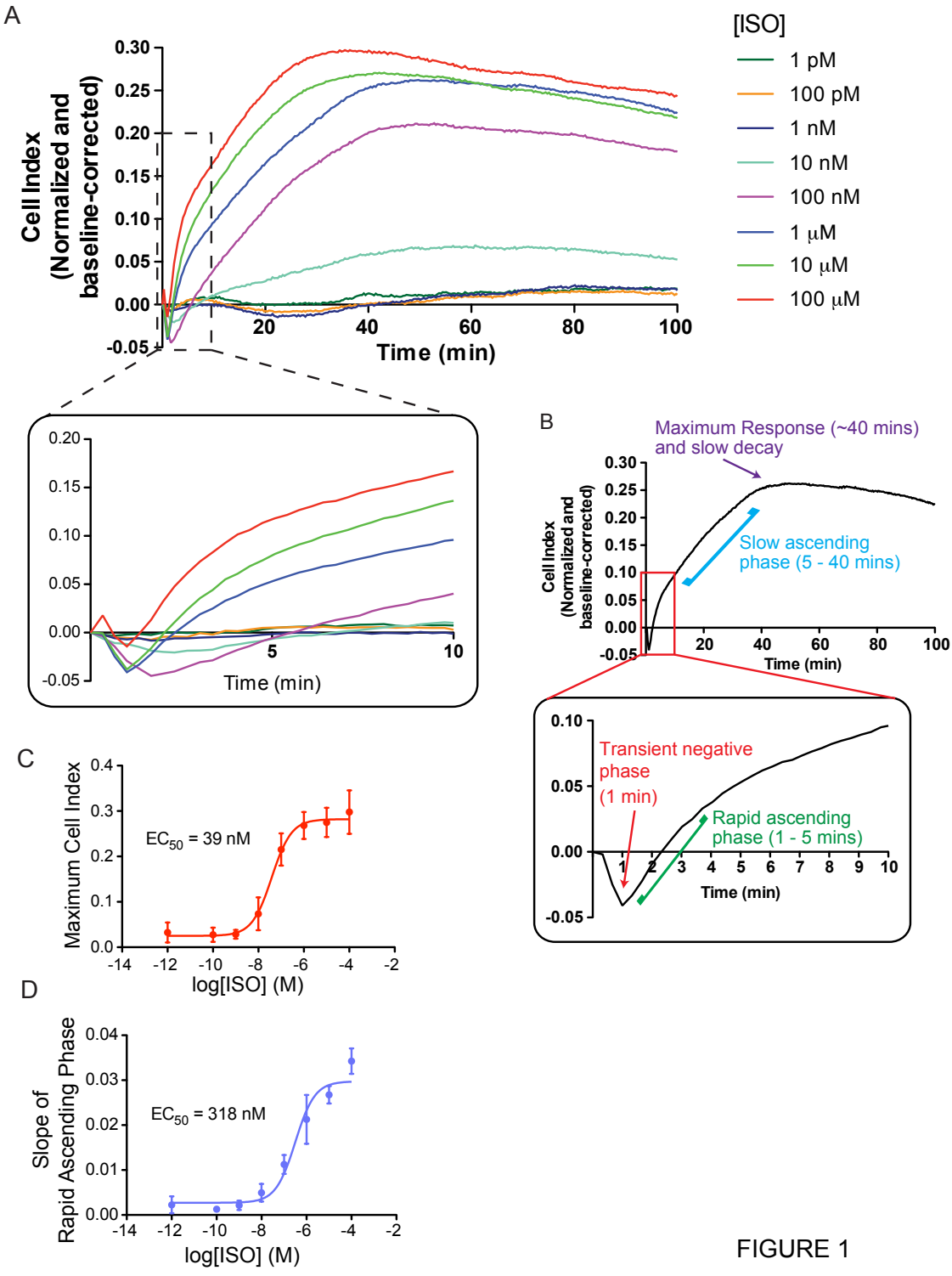

FIGURE 1

Figure 1 - Ligand-induced changes in impedance are multi-featured and concentration-dependent. (**A**) Impedance measurements were obtained in 6HisHA-β_2AR-HEK293S cells following treatment with isoproterenol (ISO) at the concentrations indicated. Impedance responses (represented as changes in Cell Index) were normalized and baseline-corrected as described in *Materials and Methods* and Figure S9. Inset: Enlargement of the transient negative phase of the response. (**B**) Features of the ISO impedance signature with the inset showing an enlargement of the first 10 minutes post-stimulation. (**C,D**) Concentration-response curves describing the maximum impedance response generated (**C**) and the slope of the rapid ascending phase (**D**) reveal distinct EC_{50} values. Data represent means of at least three independent experiments (± SEM for C and D). For clarity, SEM is not shown for impedance responses. Typical experimental variability in the impedance responses is demonstrated in Figure S5.

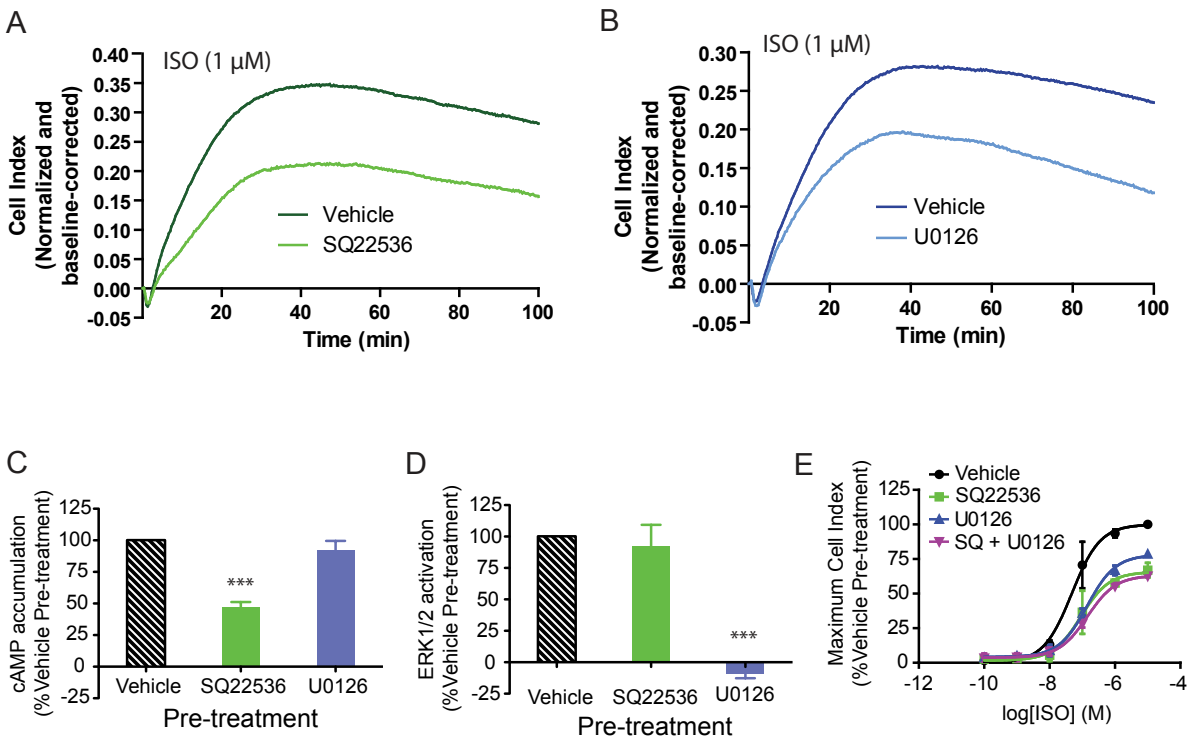

FIGURE 2

Figure 2 - Contribution of cAMP accumulation and ERK1/2 activation to the ISO-promoted impedance response. (**A,B**) Pre-treatment for 1 hour with the adenylyl cyclase inhibitor, SQ2236 (100 µM, A), or the MEK inhibitor, U0126. (5 µM, B) leads to decreases in the kinetics of the slow ascending phase and the maximum value of ISO-promoted impedance response. (**C,D**) SQ22536 and U0126 selectively inhibit ISO-promoted cAMP accumulation (C) and ERK1/2 activation (D) respectively. (**E**) Concurrent inhibition of cAMP and ERK1/2 pathways with SQ22536 and U0126 does not lead a further attenuation of the ISO-promoted maximum impedance response than either inhibitor alone. Data represent means of at least three independent experiments (± SEM for C-E). For statistical analysis, individual conditions were compared in **C** and **D** using a one-way ANOVA and a Bonferroni post-hoc test. *** $P < 0.001$.

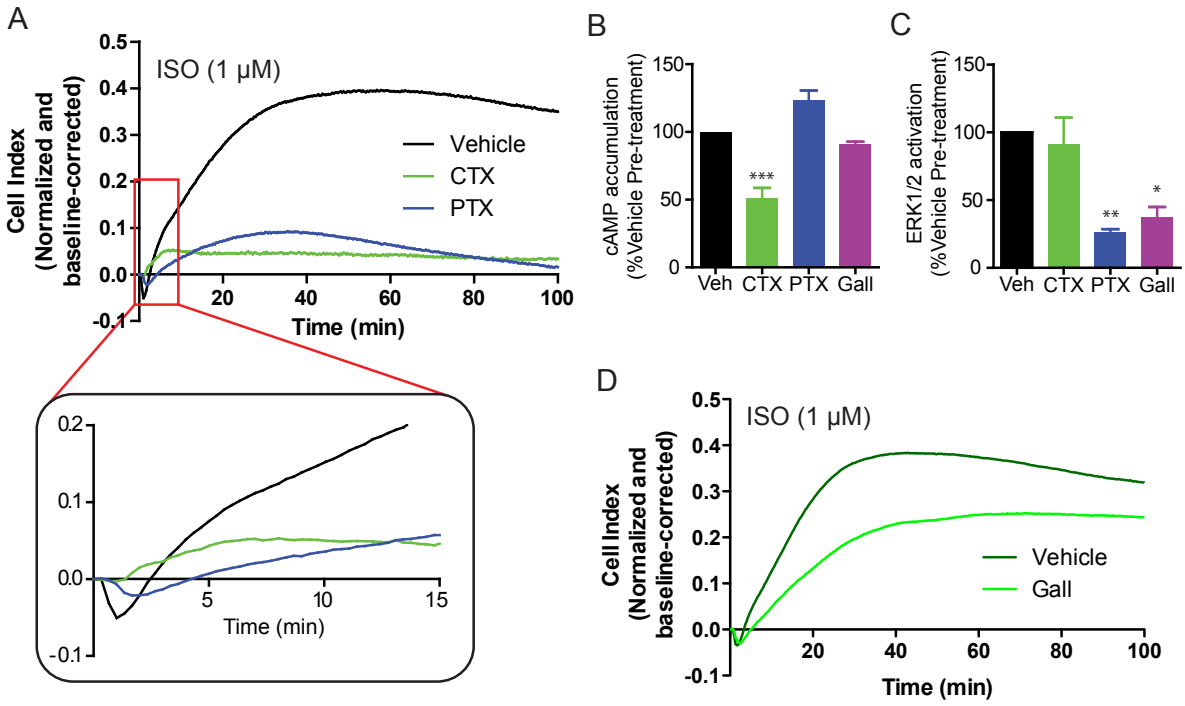

FIGURE 3

Figure 3 - Contribution of G_S, G_i, and Gβ/γ to the ISO-promoted impedance response. (**A**) Inhibition of G_S- and G_i-dependent signaling by 16 hour treatment with cholera toxin (CTX, 200 ng/ml) and pertussis toxin (PTX, 100 ng/ml), respectively, leads to distinct changes in the ISO-promoted impedance response. Inset: Enlargement of the transient negative phase of the response. (**B,C**) Effects of CTX, PTX or gallein (Gall, 100 µM, 1 hour pre-treatment) on ISO-promoted cAMP (B) and ERK1/2 responses (C).(**D**) Inhibition of Gβγ-dependent signaling by Gall leads to decreases in the kinetics of the slow ascending phase and maximum value of ISO-promoted impedance response. Data represent means of at least three independent experiments (± SEM for B and C). For statistical analysis, individual conditions were compared in **B** and **C** using a one-way ANOVA and a Bonferroni post-hoc test. *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

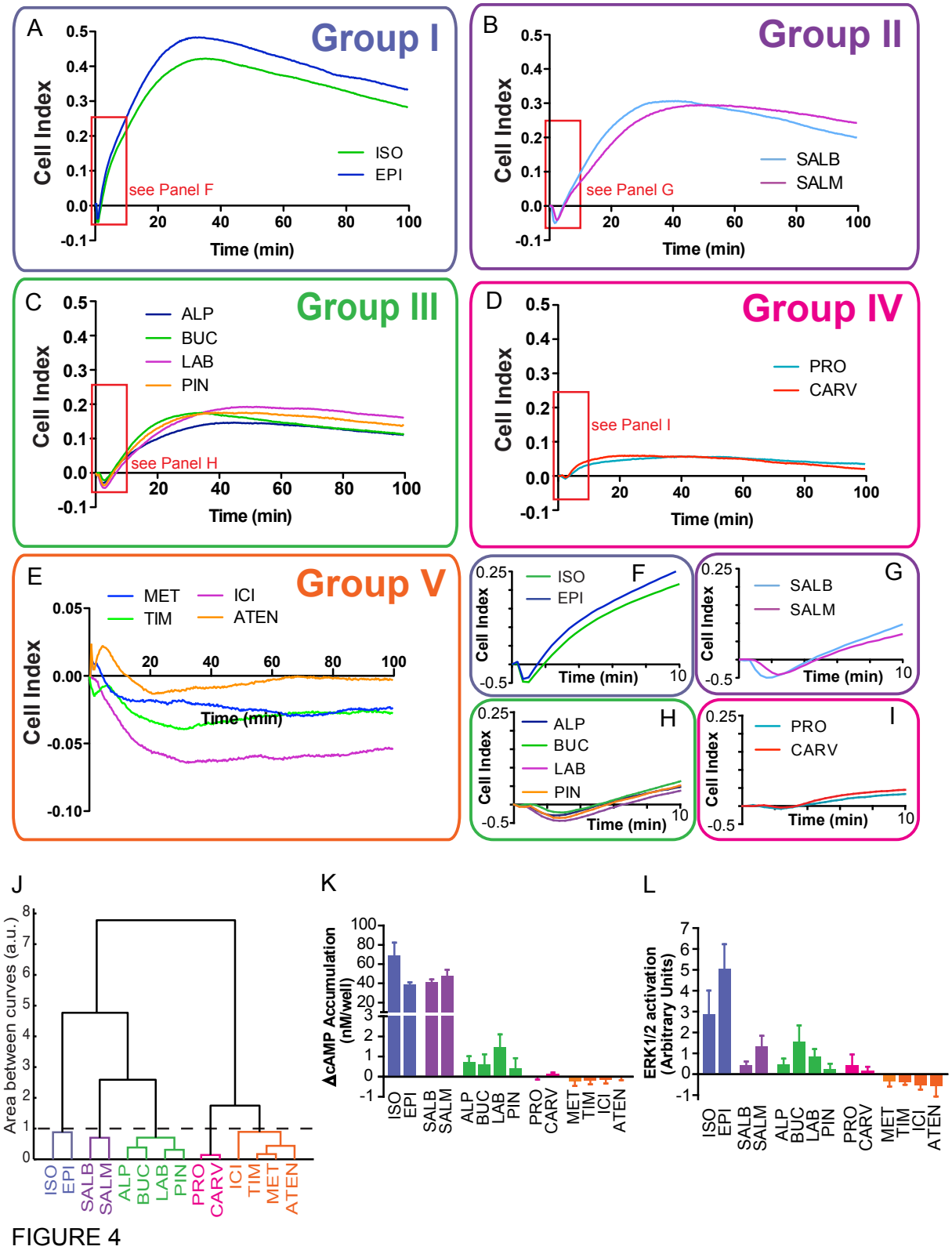
A
Group I
Cell Index
Time (min)
see Panel F
ISO
EPI
B
Group II
see Panel G
SALB
SALM
C
Group III
ALP
BUC
LAB
PIN
see Panel H
D
Group IV
PRO
CARV
see Panel I
E
Group V
MET
ICI
TIM
ATEN
F
G
H
I
J
Area between curves (a.u.)
ISO EPI SALB SALM ALP BUC LAB PIN PRO CARV ICI TIM MET ATEN
K
ΔcAMP Accumulation (nM/well)
L
ERK1/2 activation (Arbitrary Units)

FIGURE 4

Figure 4 – Impedance signatures reveal 5 distinct compound classes among β-adrenergic ligands. (**A-E**) Impedance responses were obtained following stimulation with saturating concentrations of the following ligands: ISO (50 μM), epinephrine (EPI, 75 μM), salbutamol (SALB, 100 μM), salmeterol (SALM, 1 μM), alprenolol (ALP, 1 μM), bucindolol (BUC, 1 μM), labetalol (LAB, 1 μM), pindolol (PIN, 1 μM), propranolol (PRO, 1 μM), carvedilol (CARV, 1 μM), metoprolol (MET, 20 μM), timolol (TIM, 1 μM), ICI118,551 (ICI, 1 μM) and atenolol (ATEN, 100 μM). Impedance responses, as represented by the Cell Index, were normalized and baseline-corrected. The first 10 minutes of the impedance responses are enlarged in panels **F-I**. Ligands were categorized according to complete linkage hierarchical clustering (see *Materials and Methods*) determined by comparing the area between individual curves (**J**). The dashed line represents the calculated threshold value defining compound dissimilarity. (**K,L**) Efficacies of compounds for cAMP (K) and ERK1/2 (L) pathways performed in conditions identical to those used for impedance measurements in (A-E). Data represent means of at least three independent experiments (± SEM for K and L).

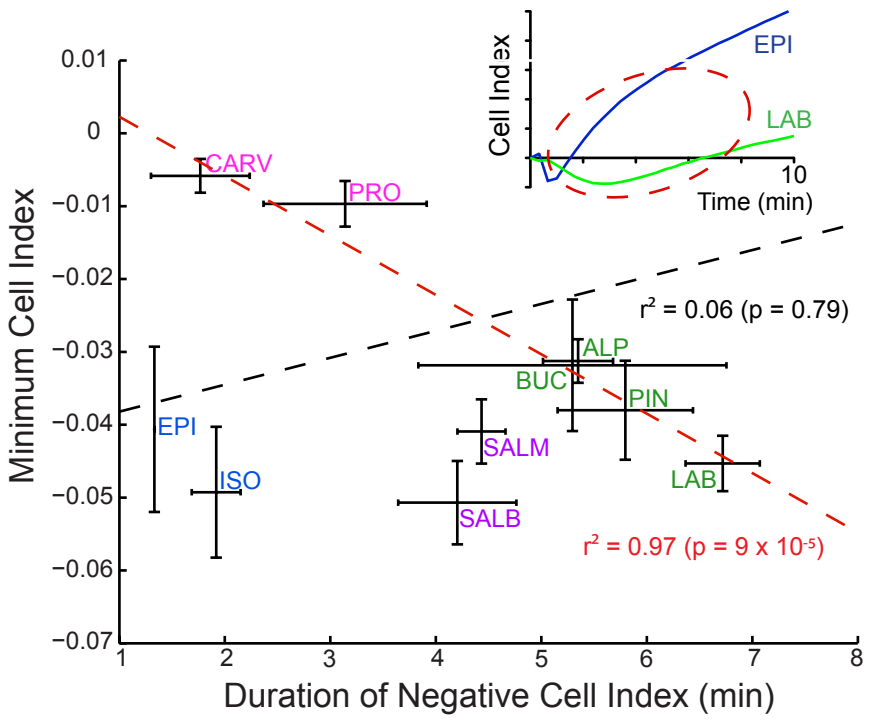

FIGURE 5

Figure 5 – Analysis of the transient negative phase of the impedance response reveals differences between Group I and II vs. Group III and IV ligands. The duration of the negative phase was plotted as a function of the minimum value for all ligands promoting a transient negative response (Group I-IV). Compound classes are indicated by color: Group I – blue, Group II – purple, Group III – green, and Group IV – pink. Lines of best fit were determined using weighted total least squares regression for all ligands (black dotted line, $r^2 = 0.06$, P = 0.79) and for Group III and IV compounds only (red dotted line, $r^2 = 0.97$, $P = 9.0 \times 10^{-5}$). Inset: The comparison of transient negative phases promoted by the prototypical Group I and Group III ligands EPI (dark blue) and LAB (green), respectively. Data represent means of at least three independent experiments (± SEM).

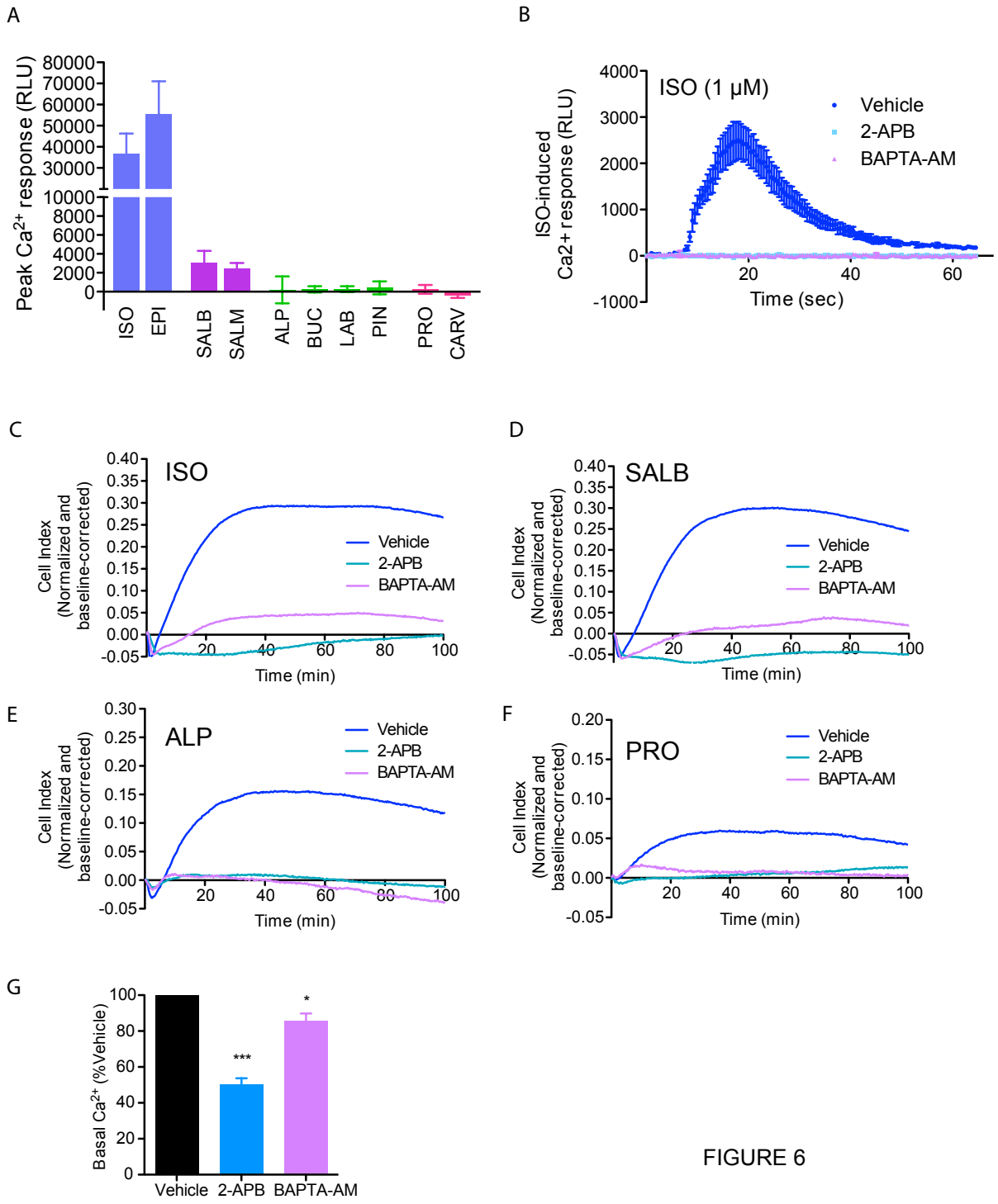
A
Peak Ca2+ response (RLU)
80000
70000
60000
50000
40000
30000
10000
8000
6000
4000
2000
0
ISO
EPI
SALB
SALM
ALP
BUC
LAB
PIN
PRO
CARV
B
ISO (1 μM)
Vehicle
2-APB
BAPTA-AM
ISO-induced Ca2+ response (RLU)
4000
3000
2000
1000
0
-1000
20
40
60
Time (sec)
C
ISO
Cell Index (Normalized and baseline-corrected)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
20
40
60
80
100
Time (min)
Vehicle
2-APB
BAPTA-AM
D
SALB
Cell Index (Normalized and baseline-corrected)
Time (min)
Vehicle
2-APB
BAPTA-AM
E
ALP
Cell Index (Normalized and baseline-corrected)
Time (min)
Vehicle
2-APB
BAPTA-AM
F
PRO
Cell Index (Normalized and baseline-corrected)
0.20
0.15
0.10
0.05
0.00
-0.05
Time (min)
Vehicle
2-APB
BAPTA-AM
G
Basal Ca2+ (%Vehicle)
100
80
60
40
20
0
***
*
Vehicle
2-APB
BAPTA-AM

FIGURE 6

Figure 6 – The role of intracellular Ca^{2+}in the impedance response to β_2AR stimulation. (**A**) Peak Ca^{2+} responses generated upon stimulation with the indicated ligand (1 µM each). The intracellular Ca^{2+} concentration is represented in relative luminescence units (RLU) emitted by the obelin biosensor (see *Materials and Methods*). (**B**) Pre-treatment for 1 hour with the IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB, 200 µM) or the intracellular Ca^{2+} chelator BAPTA-AM (20 µM) completely abolishes the ISO-promoted Ca^{2+} response. (**C-F**) Pre-treatment with 2-APB or BAPTA-AM also leads to an inhibition of the rapid ascending phase and a decrease in the maximum impedance responses of ligands from Groups I to IV (1 µM each): ISO (C), salbutamol (SALB, D), alprenolol (ALP, E) and propranolol (PRO, F), respectively. (**G**) Pre-treatment with 2-APB or BAPTA-AM significantly decreases basal intracellular [Ca^{2+}]. Data represent means of at least three independent experiments (± SEM for A, B and G). For statistical analysis, individual conditions were compared in **G** using a one-way ANOVA and a Bonferroni post-hoc test. *** $P < 0.001$ and * $P < 0.05$.

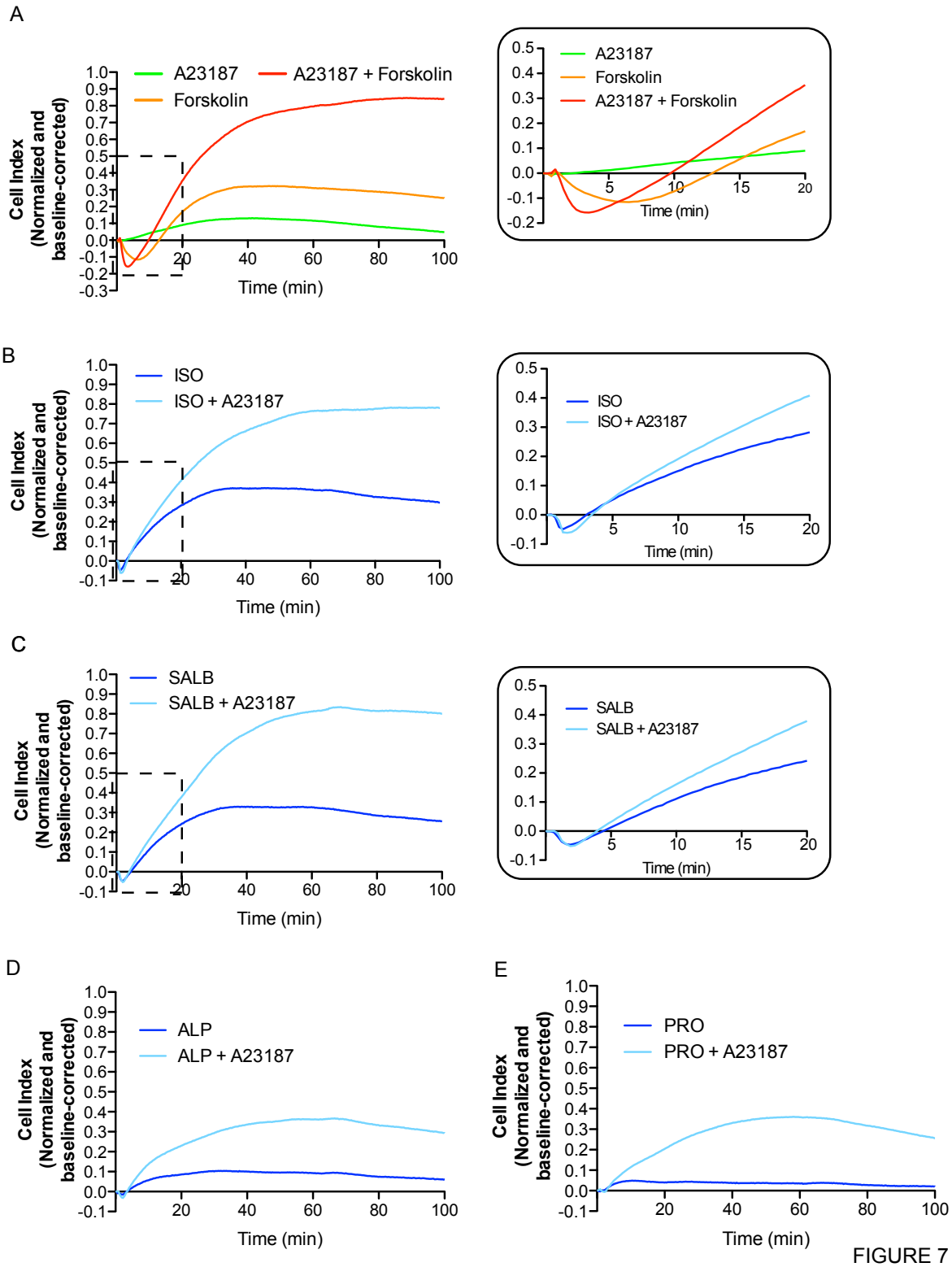
A
A23187
A23187 + Forskolin
Forskolin
Cell Index (Normalized and baseline-corrected)
Time (min)
B
ISO
ISO + A23187
C
SALB
SALB + A23187
D
ALP
ALP + A23187
E
PRO
PRO + A23187

FIGURE 7

Figure 7 – Increasing intracellular [Ca^{2+}] accelerates the rapid ascending phase and maximum impedance response. (**A**) Impedance responses obtained following treatment with the Ca^{2+} ionophore A23187 (1 μM), the adenylyl cyclase activator forskolin (10 μM) or the combined stimulation with both. (**B-E**) Impedance responses obtained following stimulation with ISO (B), SALB (C), ALP (D) and PRO (E) (1 μM each) in the presence or absence of A23187 (1 μM). Data represent means of at least three independent experiments.

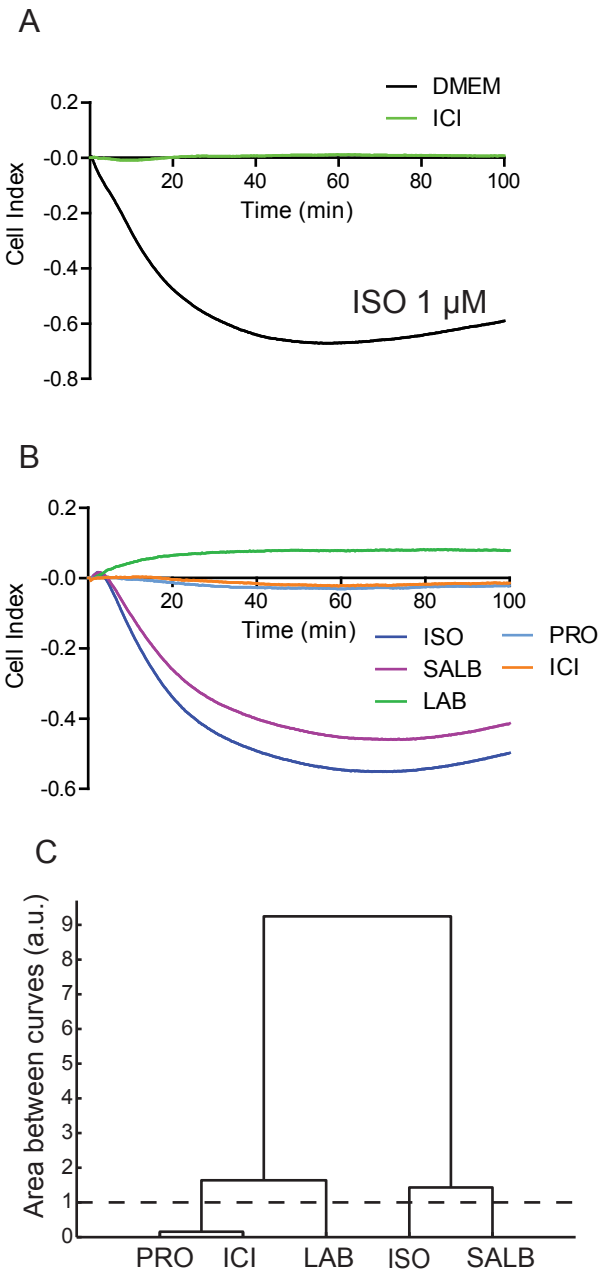

Figure 8 - β-adrenergic ligand impedance responses in rat aortic vascular smooth muscle cells (VSMCs). (**A**) Pre-treatment with the β_2-selective antagonist ICI118,551 (100 nM) for 1 hour completely abolishes the impedance response obtained stimulation with 1 µM ISO in VSMCs. (**B**) Impedance signatures for β-adrenergic ligands representing each of the 5 compound classes defined in 6HisHA-β_2AR-HEKS cells. (**C**) Complete linkage hierarchical clustering of ligand impedance responses determined by comparing the area between individual curves (see *Materials and Methods*). The dashed line represents the calculated threshold value defining compound dissimilarity. Data represent the means from at least three independent experiments.

FIGURE 8

Supporting Information

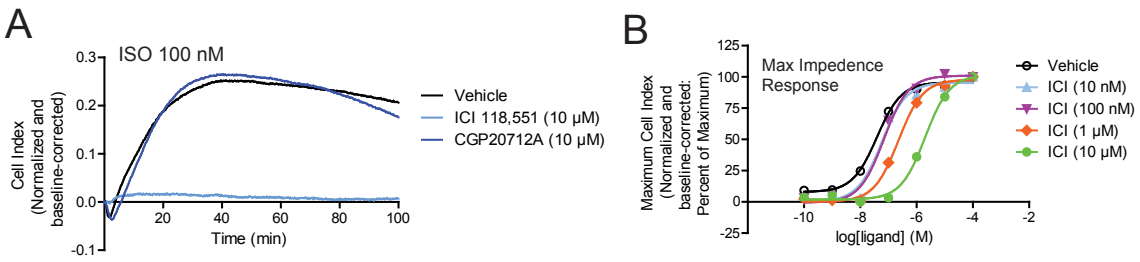

Figure S1.

Figure S1. ISO-promoted impedance response is β_2AR-specific. (**A**) Impedance response upon ISO stimulation following 1 hour pre-treatment with the β_2AR-selective antagonist ICI118,551 (ICI) or the β_1AR-selective antagonist CGP20712A (10 µM each). **(B)** Concentration-response curves of ISO-promoted maximum impedance response following pre-treatment with increasing concentrations of ICI118,551. Data represent the means from three independent experiments (+/- SEM for B).

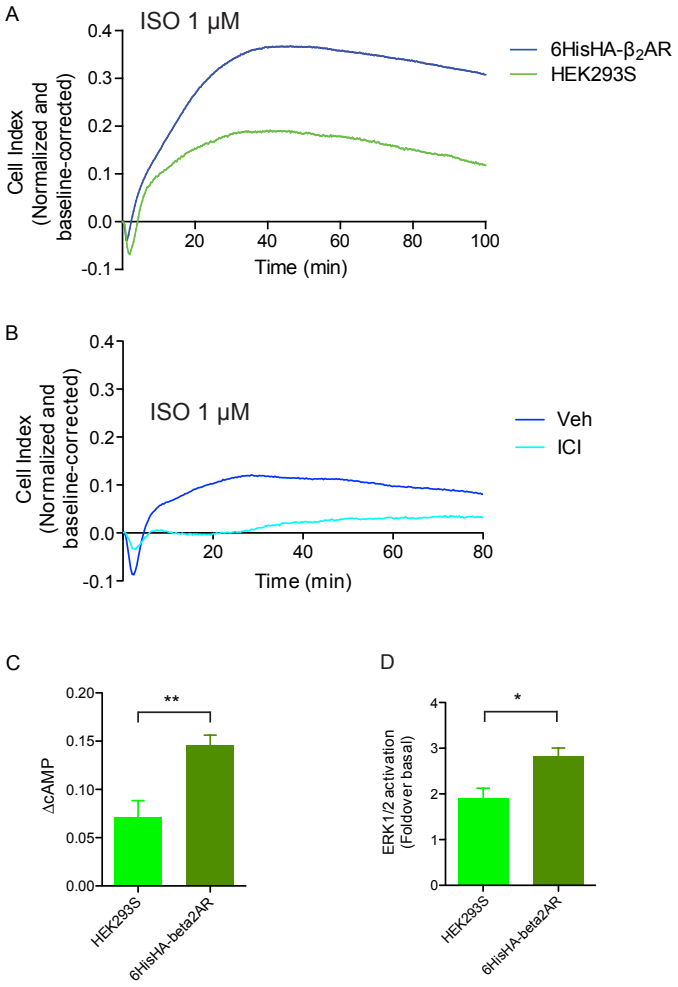

FIGURE S2

Figure S2. Signaling and impedance responses in parental HEK293S and 6HisHA-β$_2$AR-HEK293S cells. (**A**) Comparison of the impedance responses observed in parental HEK293S and 6HisHA-β$_2$AR-HEK293S stable cell lines following treatment with ISO (1 µM). (**B**) Impedance responses upon ISO stimulation in the presence or absence of a 1 hour pre-treatment with the β$_2$AR-selective antagonist ICI118,551 (ICI, 10 µM) in the parental HEK293S cells demonstrating that the response observed in these cells resulted from activation of endogenous β$_2$AR. The ISO-promoted cAMP production (**C**) and ERK1/2 activation (**D**) observed in the parental HEK293S expressing low levels of endogenous β$_2$AR was potentiated by the overexpression of human β$_2$AR in 6HisHA-β$_2$AR-HEK293S cells**.** Accumulation of cAMP was detected using the EPAC cAMP biosensor (Leduc, Breton and Heveker, *et al. J. Pharmacol. Exp. Ther.*, **331** (2009), pp. 297–307). Data represent means of at least three independent experiments. Statistical significance of the differences between the conditions for panels C and D were assessed using Student's paired *t*-test. ** $P < 0.01$, * $P < 0.05$.

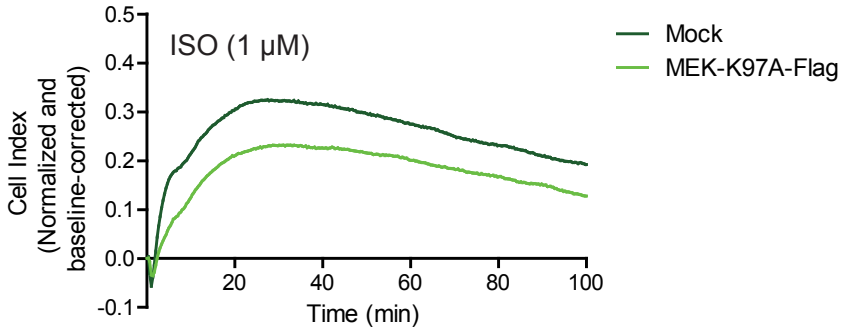

Figure S3.

Figure S3. Involvement of the MEK/ERK1/2 pathway in the ISO-promoted impedance response. Cells were transfected or not (Mock) with the MEK1-K97A-Flag dominant negative mutant 48 hours prior the impedance measurements and treated with ISO (1 µM). Data represent means of three independent experiments.

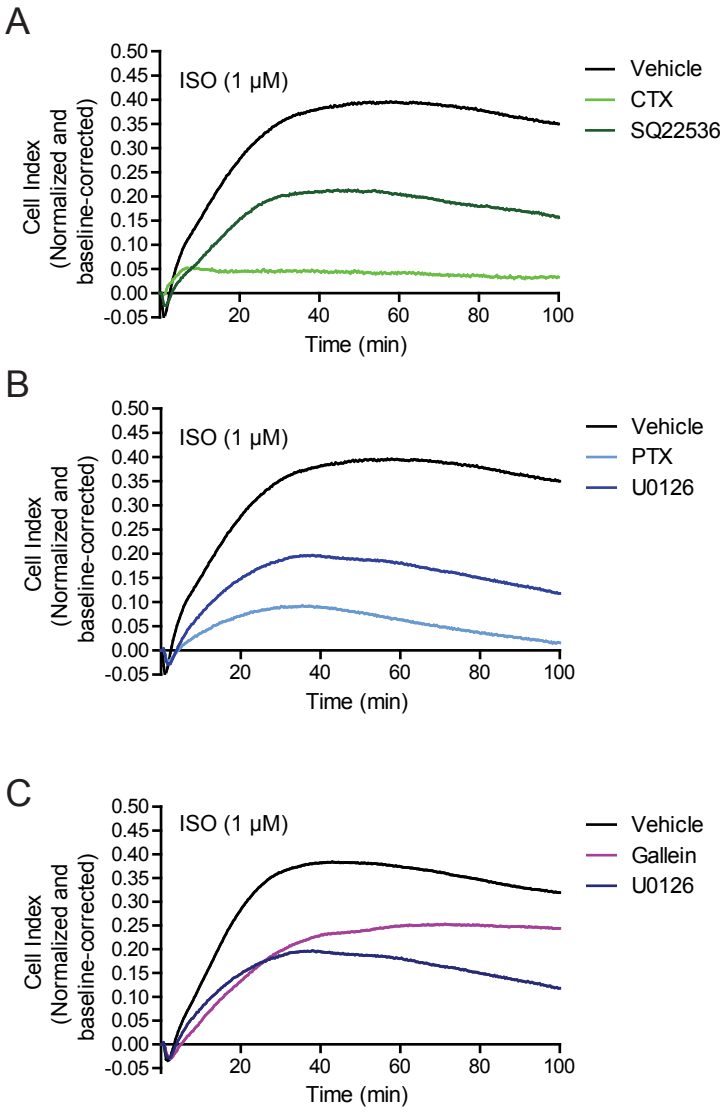

Figure S4.

Figure S4. Comparison of the direct inhibition of $G\alpha_s$, $G\alpha_i$ and $G\beta\gamma$ vs. inhibition of cAMP and ERK1/2 pathways on the impedance response. (**A**) Comparison of cholera toxin (CTX) and SQ22536 pre-treatments on the ISO-promoted impedance response (Figure 2A and 3A). (**B**) Comparison of pertussis toxin (PTX) and U0126 pre-treatments on the ISO-promoted impedance response (Figure 2B and 3A). (**C**) Comparison of gallein (Gall) and U0126 pre-treatments on the ISO-promoted impedance response (Figure 2B and 3D).

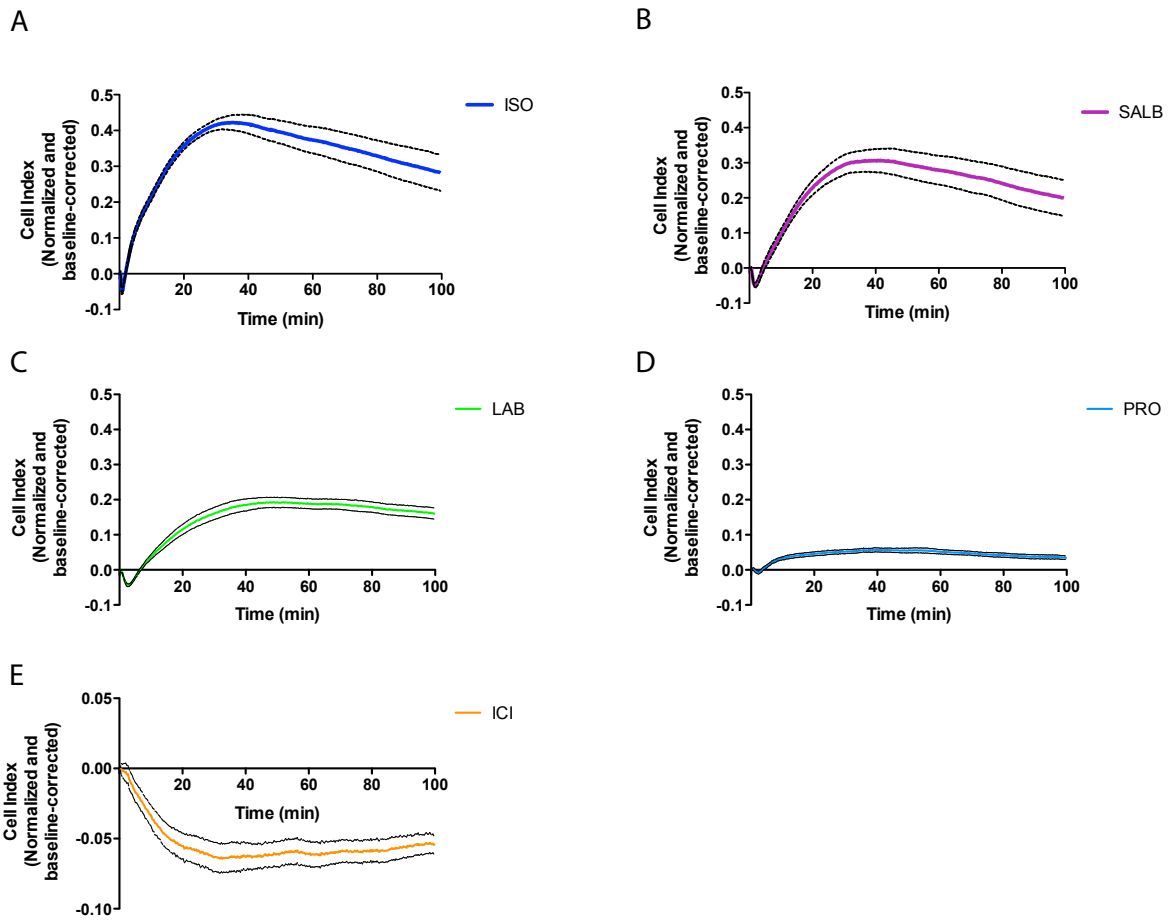

FIGURE S5

Figure S5. Statistical analysis of the variability of impedance responses. (**A-E**) Repeated impedance responses were obtained for ligands from each of the five groups of compounds. In each case, 3-7 independent measurements were made and mean impedance values (CI) were determined for each time-point. The mean impedance responses are shown in solid, colored lines whereas the dotted black lines represent the standard error of the mean (SEM) for each time-point.

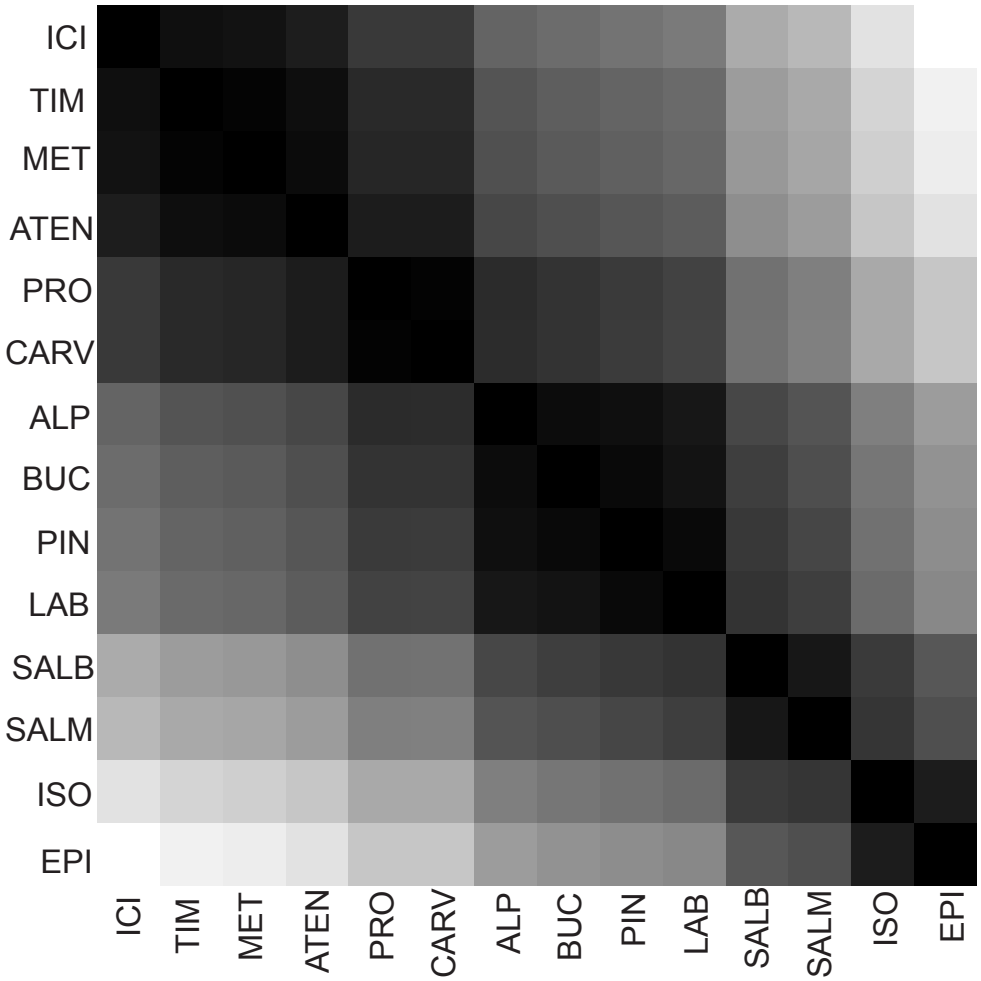

Figure S6. Visual assessment of clustering tendency of β-adrenergic ligand impedance signatures. Gray levels represent the extent of dissimilarity between ligands as determined by area between the curves, ranging from black (smallest difference) to white (largest difference). Ligands are ordered based on similarities in the area between their curves. Ligands with similar impedance signatures (small differences in area between the curves) are visualized as dark clusters along the diagonal. See *Materials and Methods* for additional information.

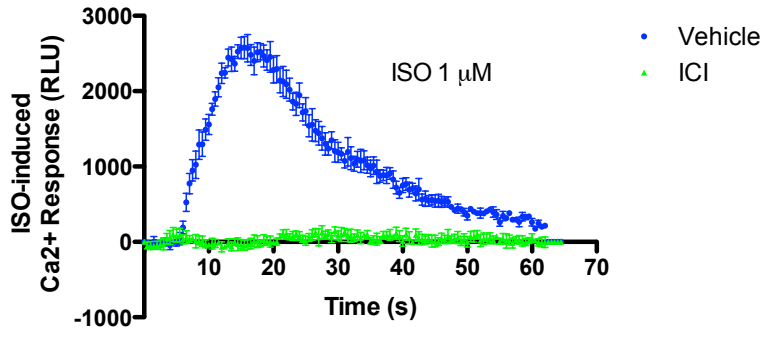

Figure S7. ISO-induced Ca^{2+} response is β_2AR-specific. 6HisHA-β_2AR-HEK293S were pre-treated or not with the β_2AR-selective antagonist ICI118,551 (100 nM) for 1 hour before stimulation with 1 μM ISO. Data represent means of three independent experiments (± SEM).

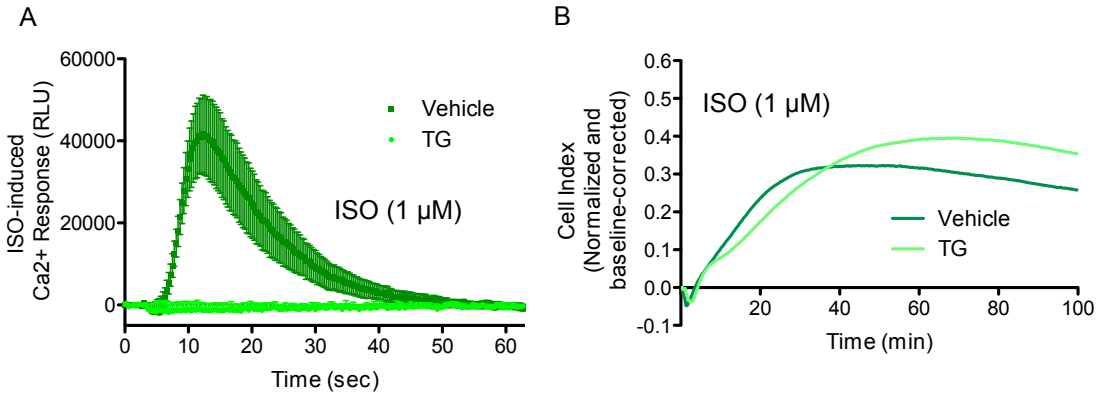

FIGURE S8

Figure S8. Effect of thapsigargin on ISO-induced Ca^{2+} and impedance responses. ISO-induced Ca^{2+} response (**A**) and impedance response (**B**) upon pre-treatment or not with thapsigargin (TG, 5 μM) for 30 minutes. Data represent means (+/- SEM for **A**) from at least three independent experiments.

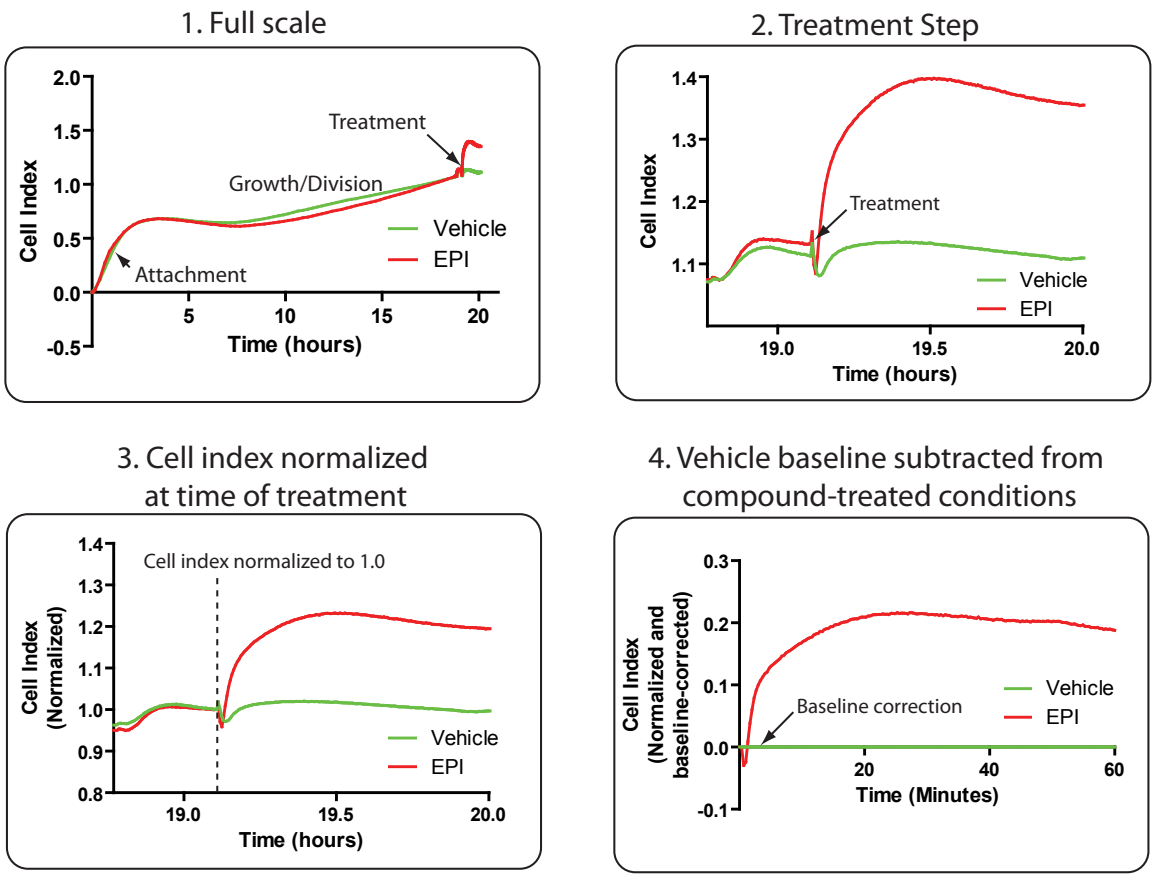

Figure S9.

Figure S9. Normalization and baseline-correction of impedance responses. (1) A measurement of cell index is made in the presence of growth medium prior to cell seeding to determine the background cell index in each well, which is subtracted from the cell index values generated by cell attachment. Cells are grown for 16-20 hours before ligand treatment. (2) Cell index values are obtained immediately following ligand treatment every 20 seconds for a total time of at least 100 minutes. (3) Cell index values are normalized by dividing by the cell index at the time of ligand addition and (4) baseline-corrected by subtracting the cell index obtained in vehicle-treated conditions. See *Materials and Methods* for more details.

ARTICLE 2

β_2 adrenergic receptors promote a Gs-dependent but cAMP-independent transactivation of P2Y11 purinergic receptors

Wayne Stallaert*, Emma van der Westhuizen* & Michel Bouvier, 2013. Finalized manuscript for submission. (*contributed equally to this work)

Context/Objectives:

This article details the mechanistic examination of the novel Ca^{2+} pathway identified in Article 1 (page 63) to contribute to the overall β_2AR cellular response. Ca^{2+} responses were assayed using a Ca^{2+}-dependent bioluminescent biosensor based on the obelin protein in HEK293S cells heterologously expressing an HA-tagged human β_2AR (HA-β_2AR-HEK293S). A rapid and transient Ca^{2+} response was detected following treatment with the prototypical β_2AR agonist isoproterenol (ISO), which was significantly inhibited upon pre-treatment with cholera toxin, implicating a role for Gs in the response observed. However, neither the adenylyl cyclase activator, forskolin, nor the protein kinase A activator, 6-Bnz-cAMP, were found to elicit a Ca^{2+} response in HA-β_2AR-HEK293S cells, suggesting that this response was cAMP-independent. Pre-treatment with the P2Y11 antagonists, suramin, NF157 and NF340 significantly inhibited the ISO-promoted Ca^{2+} response, indicating the involvement of P2Y11 receptors. Furthermore, a co-culture experiment revealed the contribution of an extracellular mediator in the ISO-promoted response. Pre-treatment with the adenosine triphosphate (ATP) diphosphohydrolase apyrase significantly inhibited ISO-promoted Ca^{2+} mobilization, identifying this extracellular mediator as ATP, the endogenous agonist of the P2Y11 receptor. The ISO-promoted Ca^{2+} response was also found to be dependent on Gq signalling and production of IP_3, implicating the preferred coupling partner of the P2Y11 receptor in the β_2AR-promoted Ca^{2+} response. Altogether, these results demonstrate that activation of the β_2AR leads to a Gs-dependent release of ATP, which transactivates the P2Y11 purinergic receptor to elicit a Gq- and IP_3-dependent mobilization of intracellular Ca^{2+}.

My contribution as co-first author included the conception and execution of many of the experiments. With my co-first author, EVW, we worked as a team on many of the experiments performed (except for the mRNA expression analysis), often each performing distinct steps of individual experiments. I also participated evenly in the analysis of the data. I wrote the first draft of the manuscript and participated greatly in its subsequent revision.

β_2 adrenergic receptors promote a Gs-dependent but cAMP-independent transactivation of P2Y11 purinergic receptors

Running title: β_2AR transactivation of P2Y11 receptors

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Key words: biosensors, G protein-coupled receptors, G protein signaling, Ca^{2+} mobilization, adrenergic receptors, purinergic receptors, receptor transactivation

ABSTRACT

The β_2 adrenergic receptor (β_2AR) has been found to couple to a variety G protein-dependent and –independent signalling pathways upon activation. In the present study, we present a novel Ca^{2+} mobilization pathway engaged by the β_2AR that involves the transactivation of P2Y11 purinergic receptors. Using a Ca^{2+}-dependent bioluminescent biosensor based on the obelin protein, a rapid and transient increase in intracellular Ca^{2+} was detected upon stimulation of HEK293S cells heterologously expressing the β_2AR (HA-β_2AR-HEK293S) with the prototypical agonist isoproterenol. While pre-treatment with cholera toxin significantly inhibited the ISO-promoted Ca^{2+} response, neither forskolin nor the protein kinase A activator 6-Bnz-cAMP were found to elicit a Ca^{2+} response, suggesting that the Ca^{2+} response in HA-β_2AR-HEK293S cells is Gs-dependent but cAMP-independent. The ISO-promoted Ca^{2+} response was also inhibited by the pan-purinergic antagonist suramin, as well as the P2Y11 antagonists NF157 and NF340, indicating the involvement of the P2Y11 receptor. Co-culture experiments revealed the role of an extracellular mediator released following ISO stimulation in the Ca^{2+} response observed. Pre-treatment with the adenosine triphosphate (ATP) diphosphohydrolase apyrase significantly inhibited ISO-promoted Ca^{2+} mobilization, indicating the identity of this extracellular mediator to be (ATP), the endogenous agonist for the P2Y11 receptor. In addition, we observed that overexpression of Gq, the dominant G protein coupling partner of the P2Y11 receptor potentiated ISO-promoted Ca^{2+} mobilization, while expression of the GQ(Q209L/D277N) dominant negative mutant abolished the response. The ISO-promoted Ca^{2+} response was also found to be inhibited upon pre-treatment with the cell-permeable Ca^{2+} chelator BAPTA-AM, the IP_3 receptor antagonist 2-aminoethoxydiphenyl borate and the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, implicating a classical Gq- and IP_3-dependent release of intracellular Ca^{2+}. Altogether, these data support a mechanism whereby the β_2AR stimulates a Gs-dependent release of ATP, which transactivates Gq-coupled P2Y11 receptors leading to an IP_3-dependent Ca^{2+}mobilization from intracellular stores.

INTRODUCTION:

β_2 adrenergic receptors couple to a variety of effectors to elicit multiple signalling events in the cell, including production of cAMP, activation of the ERK1/2 and Akt pathways, modulation of NF-κB-dependent gene transcription and regulation of cytokine interleukin (IL)-6 release (C. Chen et al., 2012; Galandrin & Bouvier, 2006; Gao et al., 2004; Zhang et al., 2011) The β_2AR has also been shown to stimulate an increase in intracellular Ca^{2+} upon activation, through several distinct mechanisms (Benitah, Alvarez, & Gómez, 2010; Schmidt et al., 2001; Tzingounis, von Zastrow, & Yudowski, 2010). Activation and integration of these signalling events underlie the important role that the β_2AR plays in various physiological processes including the regulation of cardiac function (Pérez-Schindler, Philp, & Hernandez-Cascales, 2013), smooth muscle contractility (Guimaraes & Moura, 2001), immunological responses (Sitkauskiene & Sakalauskas, 2005), fat metabolism (Collins, Cao, & Robidoux, 2004) and central nervous system activity (Nicholas, Hökfelt, & Pieribone, 1996). Because of its pleiotropic role in these many physiological processes, the β_2AR has been a major pharmacological target for a host of human pathologies for decades. Yet, despite intensive research efforts that revealed an extensive signalling repertoire for the β_2AR, new insights into its full signalling capabilities and its role in cellular biology continue to be discovered. In the current study, we follow-up on a recent report that activation of the β_2AR can lead to an increase in intracellular Ca^{2+} through an IP_3-dependent mobilization from intracellular stores (Stallaert et al 2012). Using a Ca^{2+} biosensor derived from the bioluminescent obelin protein (Markova et al., 2002), we demonstrate that this mechanism involves the β_2AR-mediated transactivation of Gq-coupled P2Y11 purinergic receptors. Activation of the β_2AR leads to a Gs-dependent but cAMP-independent extrusion of ATP, which acts in an autocrine/paracrine manner on co-expressed P2Y11 receptors. P2Y11 transactivation subsequently activates a Gq-dependent production of IP_3 and release of Ca^{2+} from intracellular stores.

METHODS:

Materials and reagents: (-)-isoproterenol hydrochloride (ISO), carbachol, thapsigargin, 6-Bnz-cAMP, forskolin, cholera toxin, pertussis toxin, suramin and apyrase were purchased from Sigma Aldrich (St Louis, MO, USA). ICI118,551, BAPTA-AM, 2-aminoethoxydiphenyl borate, SQ22536, salmeterol, NF157, NF340, NF279, A-804598, 5-BDBD and PSB-0739 were obtained from R&D Systems (Minneapolis, MN,USA). Coelenterazine CP was purchased from Biotium (Hayward, CA, USA). Cell culture reagents were from Wisent Incorporated (Montreal, QC, Canada). The Gq and Gq(Q209L/D277N) constructs were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA). All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

mCherry-obelin biosensor construct: The obelin biosensor consists of an amino-terminal mCherry joined by 5 amino acid residue linker (GSAGT) to the obelin Ca^{2+}-activated photoprotein (Markova et al., 2002). The obelin biosensor is a bioluminescent photoprotein derived from *Obelia longissima* that tightly binds the chromophore (coelenterazine) with oxygen to form a stable complex, which is activated upon binding three Ca^{2+} ions (linear range of sensitivity: 100 nM-100 µM; Illarionov et al. 2000).

Cell culture and transfection: HEK293S cells stably expressing an amino-terminal tagged human β_2AR (HA-β_2AR-HEK293S cells; Galandrin et al. 2006) were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. HA-β_2AR-HEK293S cells were transiently transfected (600 000 cells/well) in 6 well plates for obelin, bioluminescence energy transfer (BRET) and fluorescence energy transfer (FRET) assays using linear polyethylenimine (PEI; 1 mg/ml; Polysciences Inc., Warrington, PA) diluted in NaCl (150 mM, pH 7.0) (PEI:DNA ratio 3:1) as previously described (Reed, Staley, Mayginnes, Pintel, & Tullis, 2006).

Obelin Ca^{2+} measurements: HEK293S or HA-β_2AR-HEK293S cells were transiently transfected with mCherry-Obelin (950 ng/1×10^6 cells) using linear PEI as described above. Cells were re-plated (50,000 cells/well) onto white 96-well CulturePlates (Perkin-Elmer,

Woodbridge, ON, Canada) 24 h post-transfection. Growth medium was changed for reduced serum DMEM (0.5% FBS) for 18 h prior to obelin measurements. Cells were washed twice and pre-incubated in stimulation buffer (Modified Hank's Balances Salt Solution (HBSS): 137 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 0.2% (w/v) D-glucose, pH 7.4) with the obelin substrate, coelenterazine cp, (1 μM) at 25°C for 2 h in the dark. Under basal conditions, a low level of Ca^{2+}-independent luminescence is observed (Illarionov et al., 2000). Upon Ca^{2+} binding, the photoprotein oxidises coelenterazine cp, converting it to coelenteramide, releasing carbon dioxide and light in the blue range (465-495 nm). Therefore, an increase in intracellular Ca^{2+} is reflected by an increase in observed luminescence. Compounds, diluted in stimulation buffer were injected into the wells, and luminescence readings were taken every 0.3 seconds using the SpectraMax L (Molecular Devices, Sunnyvale, CA). Full kinetics were determined for each concentration of ligand for a total of 60 seconds and concentration-response curves were determined from the peak calcium responses.

cAMP production: HA-β_2AR-HEK293S were transfected with GFP_{10}-mutEPAC1(dDEP; T781A; F782A)-venus (henceforth referred to as EPAC biosensor), which consists of an amino-terminal-green fluorescent protein (GFP_{10}) joined by a 5 amino acid residue (GSAGT) linker to a mutated EPAC1(dDEP; T781A; F782A) biosensor and a carboxy-terminal-venus fluorescent protein joined by a 5 amino acid residue linker (KLPAT) (van der Westhuizen et al., 2013, *submitted for publication*). Cells were re-plated into white 96 well CulturePlates (Perkin Elmer) (50,000 cells/well) and serum starved (DMEM, 0.5% FBS) for 18 h prior to stimulation. Cells were washed 2x with stimulation buffer (modified HBSS, see above). Compounds were diluted in stimulation buffer and added to the wells at 37°C for 30 minutes prior to measurement. Under basal conditions, the GFP_{10} and venus fluorescent proteins are within close proximity, such that upon excitation of the GFP_{10} with a laser at 400nm, FRET occurs between GFP_{10} and venus. The light emitted from both fluorescent proteins was measured using the FlexStation II (Molecular Devices, Sunnyvale, CA, USA) with emission filters set at 510 nm (GFP_{10}) and 533 nm (venus) and the FRET ratio calculated (venus emission over GFP_{10} emission). Upon activation of the EPAC

biosensor, a conformational change within the biosensor causes GFP_{10} and venus to move away from each other, decreasing the FRET between these proteins. Thus, increases in intracellular cAMP levels are detected as a decrease in the FRET ratio, whereas decreases in the intracellular cAMP levels result in an increase in the FRET ratio.

Gs activation assay: HA-β_2AR-HEK293S cells were co-transfected with Gs-67-RlucII (50 ng/well), Gβ1 (100 ng/well) and GFP_{10}-Gγ1 (100 ng/well) using linear PEI as described above. Cells were re-plated (50 000 cells/well) 24 h post-transfection into white 96 well CulturePlates (Perkin Elmer). Cells were washed 2x with stimulation buffer (modified HBSS, see above), then pre-treated with inhibitors diluted in stimulation buffer for 60 minutes at 37°C. Coelenterazine 400a, diluted in stimulation buffer (5 μM) was added to the wells for 5 minutes, then increasing concentrations of ISO, diluted in stimulation buffer, were added to the wells for 2 minutes. Plates were read on the Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany), with filters set at 410±70 nm (RlucII) and 515±20 nm (GFP_{10}) and BRET ratios were calculated as GFP_{10}/RlucII.

mRNA expression analysis: Total RNA was isolated from $3x10^6$ HA-β_2AR-HEK293S cells using the RNAeasy Mini kit (Gibco, catalog # 74104) according to the manufacturer's protocol for animal cells. Microarray analysis of mRNA samples was performed using Illumina's HumanRef-8 v3.0 Expression bead chips, at McGill University and the Génome Québec Innovation Centre. The median of for the normalized data is 7.162; the minimum value is 5.851 and the maximum value of 15.760.

Data Analysis: Data analysis was performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and GraphPad Prism 5 and 6 (GraphPad Software, La Jolla, CA, USA)

RESULTS:

β_2AR activation leads to an increase in intracellular Ca^{2+} in HEK293S cells

A rapid and transient increase in intracellular Ca^{2+} was observed upon stimulation of endogenous β_2AR in HEK293S cells with the prototypical β_2AR agonist isoproterenol (ISO) (**Figure 1A**). A rapid increase in Ca^{2+} is detected 5 seconds following ISO injection, reaching a maximum at approximately 10 seconds followed by a slow decrease thereafter to return to basal levels 60 seconds post-injection. Stable overexpression of the β_2AR significantly increased this Ca^{2+} response, leading to a 3-fold increase in the magnitude of the response (**Figure 1A**). The ISO-promoted increase in Ca^{2+} was inhibited in both cell lines following pre-treatment with the β_2AR-selective antagonist, ICI 118,551 (**Figure 1B**), validating the role of the β_2AR in the Ca^{2+} response observed. This ISO-promoted response was found to be concentration-dependent, with pEC_{50} values of 7.66±0.16 and 7.12±0.24 for HEK293S and HA-β_2AR-HEK293S cells, respectively (**Figure 1C**), which is consistent with its known affinity for the β_2AR (Baker, 2010).

β_2AR promotes Ca^{2+} mobilization from intracellular stores.

The β_2AR has previously been shown to mobilize Ca^{2+} from intracellular stores (Schmidt et al., 2001; Stallaert, Dorn, van der Westhuizen, Audet, & Bouvier, 2012). Similarly, we found that the ISO-promoted Ca^{2+} response in HA-β_2AR-HEK293S cells could be completely inhibited following pre-treatment with the IP_3 receptor antagonist, 2-aminoethoxydiphenyl borate (2-APB), the cell-permeable Ca^{2+} chelator, BAPTA-AM or the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (TG) (**Figure 2A**), suggesting that ISO promotes a Ca^{2+} mobilization from intracellular stores. Similar effects were observed following treatment with the non-selective muscarinic agonist carbachol (Cch), which activates a well-characterized IP_3-dependent pathway leading to mobilization of intracellular Ca^{2+} (Caulfield, 1993) (**Figure 2B**).

ISO-stimulated Ca^{2+} response is Gs-dependent but does not involve cAMP.

In cardiomyocytes, the β_2AR is known to increase intracellular Ca^{2+} through a cAMP and protein kinase A (PKA)-dependent phosphorylation of L-type Ca^{2+} channels (Benitah et al., 2010). We therefore assessed the contribution of cAMP and PKA to the ISO-promoted response. The AC activator forskolin, despite its ability to increase cAMP to a similar extent as the β_2AR (**Figure 3A**), elicits only a very weak Ca^{2+} response (**Figure 3B**), further supporting a mechanism that does not involve cAMP. Furthermore, no detectable Ca^{2+} response was observed following treatment of cells with the PKA-activator 6-Bnz-cAMP (**Figure 3C**). We also observed that salmeterol, a β_2AR agonist that promotes cAMP production to a similar extent as ISO in HA-β_2AR-HEK293S cells (**Figure 3D**), stimulates significantly less Ca^{2+} mobilization (**Figure 3E**), further demonstrating that β_2AR-promoted cAMP production and Ca^{2+} mobilization are independent signalling events.

Since the β_2AR is traditionally classified as a Gs-coupled receptor, we investigated the contribution of this G protein to the ISO-promoted Ca^{2+} response. Following chronic pre-treatment with cholera toxin (CTX), which downregulates Gs (Levis & Bourne, 1992) or acute pre-treatment with the Gs guanine nucleotide exchange inhibitor NF449 (Hohenegger et al., 1998), we observe a significant inhibition of the ISO-promoted Ca^{2+} response (**Figure 4A**). The Cch-promoted response, despite activating a similar IP_3-dependent mobilization from intracellular stores, was not inhibited following CTX or NF449 pre-treatment (**Figure 4B**), suggesting a Gs-dependent Ca^{2+} mobilization mechanism for the β_2AR. To confirm that CTX and NF449 were indeed inhibiting the ISO-promoted Ca^{2+} response by directly inhibiting the activation of Gs, these inhibitors were tested using a bioluminescence resonance energy transfer (BRET)-based Gs activation biosensor. Interestingly, while CTX completely blocked ISO-promoted Gs activation, NF449 had no effect (**Figure 4C**), demonstrating that although the inhibition of the ISO-stimulated Ca^{2+} response by CTX results from its activity on Gs, the effect of NF449 was not dependent on its ability to directly block Gs but instead through an alternative mechanism.

Together these results suggest that the β_2AR activates the mobilization of Ca^{2+} in HA-β_2AR-HEK293S cells in a Gs-dependent but cAMP-independent manner.

Involvement of purinergic receptors.

In addition to its proposed ability to inhibit Gs activation, NF449 is also reported to be a potent antagonist of purinergic P2X and P2Y receptors (Braun et al., 2001; Kassack et al., 2004). We therefore hypothesized that since NF449 blocked ISO-promoted Ca^{2+} mobilization but did inhibit the activation of Gs (**Figure 4**), its action might be through the antagonism of purinergic receptors endogenously expressed in the HA-β_2AR-HEK293S cells. Indeed, stimulation with the purinergic agonist adenosine triphosphate (ATP) led to a rapid and transient increase in intracellular Ca^{2+} that was blocked by the pan-purinergic antagonist suramin (**Figure 5A**). Pre-treatment with suramin also significantly inhibited ISO-promoted Ca^{2+} mobilization, consistent with the potential contribution of purinergic receptors in the β_2AR response (**Figure 5B**).

To determine the purinergic receptor subtype involved in the β_2AR response, we assessed several P2X and P2Y receptor antagonists with distinct subtype selectivities. An mRNA expression analysis revealed that the most highly expressed purinergic receptor subtype in HA-β_2AR-HEK293S cells is the metabotropic P2Y11 receptor (**Supplementary Figure 1**). Pre-treatment of cells with the P2Y11/P2X1-selective antagonist NF157 or the P2Y11-selective antagonist NF340 significantly inhibited the ISO-promoted Ca^{2+} response. Furthermore, neither NF279 (P2X1-selective), PSB-0739 (P2Y12-selective), A804598 (P2X7-selective), nor 5-BDBD (P2X4-selective) were found to inhibit the response (**Figure 5B**) suggesting that ISO stimulation of the β_2AR may lead to the transactivation of the P2Y11 subtype of purinergic receptors in HA-β_2AR-HEK293S cells. NF340 did not block the Cch-promoted Ca^{2+} response, indicating that its effect does not involve an inhibition of other effectors in the Ca^{2+} mobilization pathway **(Figure 5C)**. In addition, NF340 was found to have no effect on ISO-promoted Gs activation through the β_2AR (**Figure 5D**), demonstrating that its effect also does not occur through an inhibition of Gs or blockade of the β_2AR. NF340 decreased the Emax of the ISO-promoted Ca^{2+} response with no significant effect on the EC_{50} (**Figure 5E)**, further confirming that its effect does not occur directly through a competitive blockade of the β_2AR but instead suggesting a non-competitive antagonism of the response.

Since the P2Y11 receptor couples primarily to Gq (Qi, Kennedy, Harden, & Nicholas, 2001), we next assessed the involvement of this G protein in the β_2AR-promoted Ca^{2+} response. Overexpression of Gq in HA-β_2AR-HEK293S cells leads to a 4-fold increase in the magnitude of the ISO-promoted Ca^{2+} response while expression of the Gq(Q209L/D277N) dominant negative completely abolishes this response (**Figure 6**). These results further support the role of P2Y11 receptor transactivation and the subsequent activation of Gq in the β_2AR-mediated Ca^{2+} response.

β_2AR-promoted release of extracellular ATP.

Activation of certain GPCRs has previously been shown to lead to the release of extracellular mediators, including ATP, which subsequently bind to and transactivate other receptor types (Y. Chen et al., 2006; Kronlage et al., 2010; Seminario-Vidal et al., 2009; Sumi et al., 2010; Yang, Cheek, Westfall, & Buxton, 1994). To determine if the β_2AR transactivates the P2Y11 receptor by promoting the release of an extracellular mediator such as ATP, we performed co-culture experiments in which parental HEK293S cells transfected with the obelin Ca^{2+} biosensor (HEK293S+obelin) were co-cultured with either parental HEK293S cells or with HA-β_2AR-HEK293S cells not expressing the biosensor. A significant increase in Ca^{2+} mobilization in HEK293S+obelin cells co-cultured with HA-β_2AR-HEK293S cells was observed compared the co-cultured parental HEK293S cells (**Figure 7A**), suggesting a β_2AR-dependent release of an extracellular mediator in the Ca^{2+} response.

Finally, we sought to determine if the identity of the extracellular mediator was indeed ATP, the endogenous ligand of the P2Y11 receptor. As such, cells were pre-treated with apyrase, a cell-impermeable enzyme that rapidly catalyzes the hydrolysis of ATP to AMP. Apyrase significantly inhibited the ISO-promoted response but had no effect on Cch-promoted Ca^{2+} mobilization (**Figure 7B**), providing further evidence that the β_2AR-dependent release of ATP underlies the transactivation of P2Y11 to elicit the Ca^{2+} response.

DISCUSSION

In the present study, we describe the β_2AR-mediated transactivation of the P2Y11 purinergic receptors through a Gs-dependent release of extracellular ATP and a subsequent Gq-dependent mobilization of intracellular Ca^{2+}. Accumulating evidence suggests that receptor transactivation represents a *bona fide* signalling event for many GPCRs, leading to the transactivation of various receptor tyrosine kinases (Fischer, Giordano, Comoglio, & Ullrich, 2004; Gschwind, Zwick, Prenzel, Leserer, & Ullrich, 2001; Lee, Rajagopal, & Chao, 2002) and even other GPCRs (Y. Chen et al., 2006; Kronlage et al., 2010; Ostrom, 2000; Sumi et al., 2010). Often these responses involve the production or release of an extracellular mediator that acts as a ligand for the transactivated receptor. While the β_2AR has previously been shown to transactivate adenosine type 1 receptors via cAMP extrusion (Duarte, Menezes-Rodrigues, & Godinho, 2012), β_2AR-promoted ATP release has not been observed (Sumi et al., 2010). However, the extremely short half-life of ATP limits the autocrine/paracrine actions of ATP to an extremely rapid and highly localized response wherein local concentrations reach an effective signalling level in an area no larger than a few hundred μM (Arcuino et al., 2002; Fitz, 2007; Joseph, Buchakjian, & Dubyak, 2003). Colocalization between the β_2AR and P2Y11 in specific membrane microdomains (Kaiser, 2002) could explain both the difficulty in detecting ATP accumulation in the bulk extracellular medium (Joseph et al., 2003) and the specificity of P2Y11 receptors in the response observed.

The Gs-dependent but cAMP-independent release of ATP observed represents a novel finding both in terms receptor transactivation but also in mechanism of β_2AR-mediated Ca^{2+} mobilization. Previous studies have revealed several alternative mechanisms through which the β_2AR can regulate intracellular Ca^{2+} levels (Benitah et al., 2010; Schmidt et al., 2001; Tzingounis et al., 2010); however, these studies often implicate cAMP and its effectors PKA and EPAC. Although the current study demonstrates the key role of Gs in this response, the mechanism through which its activation leads to the release of ATP remains elusive. Interestingly, activation of β-adrenergic receptors has previously been shown to potentiate purinergic receptor-induced Ca^{2+} mobilization through a mechanism involving

Gs but not cAMP (Jiménez et al., 1999). The authors of this paper propose that this may occur via an intracellular cross-talk mechanism mediated by the βγ-subunits released upon Gs activation. However, while the ability of βγ-subunits to increase phospholipase C (PLC) activity is a well-established mechanism of several Gi-coupled receptors (Biber, Klotz, Berger, Gebicke-Harter, & van Calker, 1997; Boyer et al., 1989; Dorn, Oswald, McCluskey, Kuhel, & Liggett, 1997; Kuang, 1996; Mizuta et al., 2011; Park, Jhon, Lee, Lee, & Rhee, 1993; Thodeti, 2000), this Gβγ-mediated effect has not been explicitly demonstrated following release from Gαs subunits. Furthermore, the observation that the ATP diphosphohydrylase apyrase blocked the ISO-promoted Ca^{2+} response indicates that crosstalk between the β_2AR and the P2Y11 receptor must include an extracellular component.

We must therefore consider the role of other Gs effectors in the β_2AR-promoted ATP release. The adaptor protein tetratricopeptide repeat 1 (TPR1), has recently been identified as a novel interacting partner of Gs. While the downstream signalling consequences of the interaction between Gs and TPR1 have yet to be explored, the complex formed between TPR1 and the alpha subunit of G_{16} recruits the active form of Ras and modulates ERK1/2 activation (Marty, Browning, & Ye, 2003). Given the ability of Ras to interact with certain PLC isoforms to elicit IP_3 production (Seifert, Zhou, Hicks, Sondek, & Harden, 2008), further study of the potential role of TPR-1 in the β_2AR-mediated transactivation of P2Y11 receptors could provide key mechanistic details into this novel signalling paradigm.

Given that the β_2AR and the P2Y11 receptor are co-expressed in human airway epithelial cells (Umapathy et al., 2010; Zink, Rösen, Sackmann, & Lemoine, 1993), the result obtained in the current study could have important implications in understanding how the β_2AR and P2Y receptors might collaborate in normal physiology and in the treatment of pulmonary disorders. The β_2AR plays an important physiological role in the airway epithelium where, through mechanisms involving cAMP/PKA (Barnes, 2011; Giembycz & Newton, 2006), it participates in the secretion of airway surfactants, ciliary beating and regulation of mucosal clearance (Salathe, 2002; Wright & Dobbs, 1991), properties that contribute to the therapeutic success of β_2AR agonists for the treatment of asthma (Giembycz & Newton, 2006). However, while acute administration of β_2AR agonists is highly effective in relieving

airway hypersensitivity in asthmatic patients, chronic administration has been shown to induce deleterious effects including airway hyperresponsiveness due in part to the release of inflammatory cytokines such as IL-6 and IL-8 from epithelial cells (Cheung et al., 1992; Holden et al., 2010; Lin et al., 2012). For this reason, β_2AR agonist treatment is often coupled with inhaled corticosteroid administration to repress transcription of pro-inflammatory cytokines and enhance transcription of anti-inflammatory mediators (Sin & Man, 2006). Activation of Gq-coupled receptors and increases in intracellular [Ca^{2+}], on the other hand, promote inflammatory responses in pulmonary tissues (Barnes et al 1998; Rider et al, JPET 2011). Interestingly, an increase in purine nucleotide release from the airway epithelium is a hallmark of inflammatory diseases in the lungs (Burnstock, Brouns, Adriaensen, & Timmermans, 2012) and purinergic signalling has been proposed to participate in asthmatic airway inflammation (Basoglu et al., 2005; Idzko et al., 2007) and the pathogenesis of chronic obstructive pulmonary disorder (COPD) (Adriaensen & Timmermans, 2004; Mortaz, Folkerts, Nijkamp, & Henricks, 2010).

Given the results obtained in the current study, optimal therapy for such pulmonary disorders could involve harnessing the therapeutic benefit of β-adrenergic-mediated cAMP/PKA activity while avoiding the pro-inflammatory effect of purinergic transactivation and subsequent Ca^{2+}-dependent signalling. One potential therapeutic strategy could take advantage of an emerging paradigm in GPCR pharmacology, ligand functional selectivity. In the past few years, it has become established that GPCRs often couple to multiple G protein-dependent and -independent signalling events from a given receptor subtype, and that ligands for these receptors often exhibit a selectivity towards certain pathways over others. This has generated a lot of excitement in the field of GPCR drug discovery on the basis that drugs might be found that selectively target signalling pathways underlying a given pathology without modulating unrelated, potentially detrimental pathways. In the context of the present study, the identification of a β_2AR ligand that stimulates cAMP production without significant transactivation of P2Y11 receptors could represent an ideal class of β_2AR agonist for the treatment of pulmonary disorders. Salmeterol, a clinically effective β_2AR agonist used in the treatment of asthma exhibits less side effects than what is typically associated with chronic use of β_2AR agonists

(Cazzola, Page, Rogliani, & Matera, 2013). Although these properties are believed to be related to a reduced propensity to induce receptor desensitization for salmeterol compared to other β_2AR agonists, it may be of interest to examine if the reduced P2Y11 receptor transactivation observed in the current study (**Figure 3E**) contributes to this positive clinical profile and identify agonists with an even greater bias towards cAMP production versus Ca^{2+} mobilization.

REFERENCES

Adriaensen, D., & Timmermans, J.-P. (2004). Purinergic signalling in the lung: important in asthma and COPD? *Current opinion in pharmacology*, *4*(3), 207–14. doi:10.1016/j.coph.2004.01.010

Arcuino, G., Lin, J. H.-C., Takano, T., Liu, C., Jiang, L., Gao, Q., … Nedergaard, M. (2002). Intercellular calcium signaling mediated by point-source burst release of ATP. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(15), 9840–5. doi:10.1073/pnas.152588599

Baker, J. G. (2010). The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. *British journal of pharmacology*, *160*(5), 1048–61. doi:10.1111/j.1476-5381.2010.00754.x

Barnes, P. J. (2011). Biochemical basis of asthma therapy. *The Journal of biological chemistry*, *286*(38), 32899–905. doi:10.1074/jbc.R110.206466

Basoglu, O. K., Pelleg, A., Essilfie-Quaye, S., Brindicci, C., Barnes, P. J., & Kharitonov, S. A. (2005). Effects of aerosolized adenosine 5'-triphosphate vs adenosine 5'-monophosphate on dyspnea and airway caliber in healthy nonsmokers and patients with asthma. *Chest*, *128*(4), 1905–9. doi:10.1378/chest.128.4.1905

Benitah, J.-P., Alvarez, J. L., & Gómez, A. M. (2010). L-type Ca(2+) current in ventricular cardiomyocytes. *Journal of molecular and cellular cardiology*, *48*(1), 26–36. doi:10.1016/j.yjmcc.2009.07.026

Biber, K., Klotz, K.-N., Berger, M., Gebicke-Harter, P. J., & van Calker, D. (1997). Adenosine A1 Receptor-Mediated Activation of Phospholipase C in Cultured Astrocytes Depends on the Level of Receptor Expression. *J. Neurosci.*, *17*(13), 4956–4964.

Boyer, J. L., Waldo, G. L., Evans, T., Northup, J. K., Downes, C. P., & Harden, T. K. (1989). Modification of AlF-4- and receptor-stimulated phospholipase C activity by G-protein beta gamma subunits. *The Journal of biological chemistry*, *264*(23), 13917–22.

Braun, K., Rettinger, J., Ganso, M., Kassack, M., Hildebrandt, C., Ullmann, H., … Lambrecht, G. (2001). NF449: a subnanomolar potency antagonist at recombinant rat P2X1 receptors. *Naunyn-Schmiedeberg's archives of pharmacology*, *364*(3), 285–90.

Burnstock, G., Brouns, I., Adriaensen, D., & Timmermans, J.-P. (2012). Purinergic signaling in the airways. *Pharmacological reviews*, *64*(4), 834–68. doi:10.1124/pr.111.005389

Caulfield, M. P. (1993). Muscarinic receptors--characterization, coupling and function. *Pharmacology & therapeutics*, *58*(3), 319–79.

Cazzola, M., Page, C. P., Rogliani, P., & Matera, M. G. (2013). β2-agonist therapy in lung disease. *American journal of respiratory and critical care medicine*, *187*(7), 690–6. doi:10.1164/rccm.201209-1739PP

Chen, C., Du, J., Feng, W., Song, Y., Lu, Z., Xu, M., … Zhang, Y. (2012). β-Adrenergic receptors stimulate interleukin-6 production through Epac-dependent activation of PKCδ/p38 MAPK signalling in neonatal mouse cardiac fibroblasts. *British journal of pharmacology*, *166*(2), 676–88. doi:10.1111/j.1476-5381.2011.01785.x

Chen, Y., Corriden, R., Inoue, Y., Yip, L., Hashiguchi, N., Zinkernagel, A., … Junger, W. G. (2006). ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science (New York, N.Y.)*, *314*(5806), 1792–5. doi:10.1126/science.1132559

Cheung, D., Timmers, M. C., Zwinderman, A. H., Bel, E. H., Dijkman, J. H., & Sterk, P. J. (1992). Long-term effects of a long-acting beta 2-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *The New England journal of medicine*, *327*(17), 1198–203. doi:10.1056/NEJM199210223271703

Collins, S., Cao, W., & Robidoux, J. (2004). Learning new tricks from old dogs: beta-adrenergic receptors teach new lessons on firing up adipose tissue metabolism. *Molecular endocrinology (Baltimore, Md.)*, *18*(9), 2123–31. doi:10.1210/me.2004-0193

Dorn, G. W., Oswald, K. J., McCluskey, T. S., Kuhel, D. G., & Liggett, S. B. (1997). Alpha 2A-adrenergic receptor stimulated calcium release is transduced by Gi-associated G(beta gamma)-mediated activation of phospholipase C. *Biochemistry*, *36*(21), 6415–23. doi:10.1021/bi970080s

Duarte, T., Menezes-Rodrigues, F. S., & Godinho, R. O. (2012). Contribution of the extracellular cAMP-adenosine pathway to dual coupling of β2-adrenoceptors to Gs and Gi proteins in mouse skeletal muscle. *The Journal of pharmacology and experimental therapeutics*, *341*(3), 820–8. doi:10.1124/jpet.112.192997

Fischer, O. M., Giordano, S., Comoglio, P. M., & Ullrich, A. (2004). Reactive oxygen species mediate Met receptor transactivation by G protein-coupled receptors and the epidermal growth factor receptor in human carcinoma cells. *The Journal of biological chemistry*, *279*(28), 28970–8. doi:10.1074/jbc.M402508200

Fitz, J. G. (2007). Regulation of cellular ATP release. *Transactions of the American Clinical and Climatological Association*, *118*, 199–208.

Galandrin, S., & Bouvier, M. (2006). Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol*, *70*(5), 1575–1584. doi:mol.106.026716 [pii] 10.1124/mol.106.026716

Gao, H., Sun, Y., Wu, Y., Luan, B., Wang, Y., Qu, B., & Pei, G. (2004). Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. *Molecular cell*, *14*(3), 303–17.

Giembycz, M. A., & Newton, R. (2006). Beyond the dogma: novel beta2-adrenoceptor signalling in the airways. *The European respiratory journal*, *27*(6), 1286–306. doi:10.1183/09031936.06.00112605

Gschwind, A., Zwick, E., Prenzel, N., Leserer, M., & Ullrich, A. (2001). Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene*, *20*(13), 1594–600. doi:10.1038/sj.onc.1204192

Guimaraes, S., & Moura, D. (2001). Vascular Adrenoceptors: An Update. *Pharmacol. Rev.*, *53*(2), 319–356.

Hohenegger, M., Waldhoer, M., Beindl, W., Böing, B., Kreimeyer, A., Nickel, P., … Freissmuth, M. (1998). Gsalpha-selective G protein antagonists. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(1), 346–51.

Holden, N. S., Rider, C. F., Bell, M. J., Velayudhan, J., King, E. M., Kaur, M., … Newton, R. (2010). Enhancement of inflammatory mediator release by beta(2)-adrenoceptor agonists in airway epithelial cells is reversed by glucocorticoid action. *British journal of pharmacology*, *160*(2), 410–20. doi:10.1111/j.1476-5381.2010.00708.x

Idzko, M., Hammad, H., van Nimwegen, M., Kool, M., Willart, M. A. M., Muskens, F., … Lambrecht, B. N. (2007). Extracellular ATP triggers and maintains asthmatic airway inflammation by activating dendritic cells. *Nature medicine*, *13*(8), 913–9. doi:10.1038/nm1617

Illarionov, B. A., Frank, L. A., Illarionova, V. A., Bondar, V. S., Vysotski, E. S., & Blinks, J. R. (2000). Recombinant obelin: cloning and expression of cDNA purification, and characterization as a calcium indicator. *Methods in enzymology*, *305*, 223–49.

Jiménez, A. I., Castro, E., Mirabet, M., Franco, R., Delicado, E. G., & Miras-Portugal, M. T. (1999). Potentiation of ATP calcium responses by A2B receptor stimulation and other signals coupled to Gs proteins in type-1 cerebellar astrocytes. *Glia*, *26*(2), 119–28.

Joseph, S. M., Buchakjian, M. R., & Dubyak, G. R. (2003). Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *The Journal of biological chemistry*, *278*(26), 23331–42. doi:10.1074/jbc.M302680200

Kaiser, R. A. (2002). Functional Compartmentation of Endothelial P2Y Receptor Signaling. *Circulation Research*, *91*(4), 292–299. doi:10.1161/01.RES.0000030711.21521.AC

Kassack, M. U., Braun, K., Ganso, M., Ullmann, H., Nickel, P., Böing, B., … Lambrecht, G. (2004). Structure-activity relationships of analogues of NF449 confirm NF449 as the most potent and selective known P2X1 receptor antagonist. *European journal of medicinal chemistry*, *39*(4), 345–57. doi:10.1016/j.ejmech.2004.01.007

Kronlage, M., Song, J., Sorokin, L., Isfort, K., Schwerdtle, T., Leipziger, J., … Hanley, P. J. (2010). Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. *Science signaling*, *3*(132), ra55. doi:10.1126/scisignal.2000588

Kuang, Y. (1996). Pertussis Toxin-sensitive Activation of Phospholipase C by the C5a and fMet-Leu-Phe Receptors. *Journal of Biological Chemistry*, *271*(23), 13430–13434. doi:10.1074/jbc.271.23.13430

Lee, F. S., Rajagopal, R., & Chao, M. V. (2002). Distinctive features of Trk neurotrophin receptor transactivation by G protein-coupled receptors. *Cytokine & Growth Factor Reviews*, *13*(1), 11–17. doi:10.1016/S1359-6101(01)00024-7

Levis, M. J., & Bourne, H. R. (1992). Activation of the alpha subunit of Gs in intact cells alters its abundance, rate of degradation, and membrane avidity. *The Journal of cell biology*, *119*(5), 1297–307.

Lin, R., Degan, S., Theriot, B. S., Fischer, B. M., Strachan, R. T., Liang, J., … Walker, J. K. L. (2012). Chronic treatment in vivo with β-adrenoceptor agonists induces dysfunction of airway β(2) -adrenoceptors and exacerbates lung inflammation in mice. *British journal of pharmacology*, *165*(7), 2365–77. doi:10.1111/j.1476-5381.2011.01725.x

Markova, S. V, Vysotski, E. S., Blinks, J. R., Burakova, L. P., Wang, B.-C., & Lee, J. (2002). Obelin from the bioluminescent marine hydroid Obelia geniculata: cloning, expression, and comparison of some properties with those of other Ca2+-regulated photoproteins. *Biochemistry*, *41*(7), 2227–36.

Marty, C., Browning, D. D., & Ye, R. D. (2003). Identification of tetratricopeptide repeat 1 as an adaptor protein that interacts with heterotrimeric G proteins and the small GTPase Ras. *Molecular and cellular biology*, *23*(11), 3847–58.

Mizuta, K., Mizuta, F., Xu, D., Masaki, E., Panettieri, R. A., & Emala, C. W. (2011). Gi-coupled γ-aminobutyric acid-B receptors cross-regulate phospholipase C and calcium in airway smooth muscle. *American journal of respiratory cell and molecular biology*, *45*(6), 1232–8. doi:10.1165/rcmb.2011-0088OC

Mortaz, E., Folkerts, G., Nijkamp, F. P., & Henricks, P. A. J. (2010). ATP and the pathogenesis of COPD. *European journal of pharmacology*, *638*(1-3), 1–4. doi:10.1016/j.ejphar.2010.04.019

Nicholas, A. P., Hökfelt, T., & Pieribone, V. A. (1996). The distribution and significance of CNS adrenoceptors examined with in situ hybridization. *Trends in pharmacological sciences*, *17*(7), 245–55.

Ostrom, R. S. (2000). Cellular Release of and Response to ATP as Key Determinants of the Set-Point of Signal Transduction Pathways. *Journal of Biological Chemistry*, *275*(16), 11735–11739. doi:10.1074/jbc.275.16.11735

Park, D., Jhon, D., Lee, C., Lee, K., & Rhee, S. (1993). Activation of phospholipase C isozymes by G protein beta gamma subunits. *J. Biol. Chem.*, *268*(7), 4573–4576.

Pérez-Schindler, J., Philp, A., & Hernandez-Cascales, J. (2013). Pathophysiological relevance of the cardiac β2-adrenergic receptor and its potential as a therapeutic target to improve cardiac function. *European journal of pharmacology*, *698*(1-3), 39–47. doi:10.1016/j.ejphar.2012.11.001

Qi, A. D., Kennedy, C., Harden, T. K., & Nicholas, R. A. (2001). Differential coupling of the human P2Y(11) receptor to phospholipase C and adenylyl cyclase. *British journal of pharmacology*, *132*(1), 318–26. doi:10.1038/sj.bjp.0703788

Reed, S. E., Staley, E. M., Mayginnes, J. P., Pintel, D. J., & Tullis, G. E. (2006). Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors. *Journal of virological methods*, *138*(1-2), 85–98. doi:10.1016/j.jviromet.2006.07.024

Salathe, M. (2002). Effects of β-agonists on airway epithelial cells. *Journal of Allergy and Clinical Immunology*, *110*(6), S275–S281. doi:10.1067/mai.2002.129412

Schmidt, M., Evellin, S., Weernink, P. A., von Dorp, F., Rehmann, H., Lomasney, J. W., & Jakobs, K. H. (2001). A new phospholipase-C-calcium signalling pathway mediated by cyclic AMP and a Rap GTPase. *Nature cell biology*, *3*(11), 1020–4. doi:10.1038/ncb1101-1020

Seifert, J. P., Zhou, Y., Hicks, S. N., Sondek, J., & Harden, T. K. (2008). Dual activation of phospholipase C-epsilon by Rho and Ras GTPases. *The Journal of biological chemistry*, *283*(44), 29690–8. doi:10.1074/jbc.M805038200

Seminario-Vidal, L., Kreda, S., Jones, L., O'Neal, W., Trejo, J., Boucher, R. C., & Lazarowski, E. R. (2009). Thrombin promotes release of ATP from lung epithelial cells through coordinated activation of rho- and Ca2+-dependent signaling pathways. *The Journal of biological chemistry*, *284*(31), 20638–48. doi:10.1074/jbc.M109.004762

Sin, D. D., & Man, S. F. P. (2006). Corticosteroids and adrenoceptor agonists: the compliments for combination therapy in chronic airways diseases. *European journal of pharmacology*, *533*(1-3), 28–35. doi:10.1016/j.ejphar.2005.12.049

Sitkauskiene, B., & Sakalauskas, R. (2005). The role of beta(2)-adrenergic receptors in inflammation and allergy. *Current drug targets. Inflammation and allergy*, *4*(2), 157–62.

Stallaert, W., Dorn, J. F., van der Westhuizen, E., Audet, M., & Bouvier, M. (2012). Impedance Responses Reveal β(2)-Adrenergic Receptor Signaling Pluridimensionality and Allow Classification of Ligands with Distinct Signaling Profiles. (C. V. Rao, Ed.)*PloS one*, *7*(1), e29420. doi:10.1371/journal.pone.0029420

Sumi, Y., Woehrle, T., Chen, Y., Yao, Y., Li, A., & Junger, W. G. (2010). Adrenergic receptor activation involves ATP release and feedback through purinergic receptors. *American journal of physiology. Cell physiology*, *299*(5), C1118–26. doi:10.1152/ajpcell.00122.2010

Thodeti, C. K. (2000). Leukotriene D4 Triggers an Association between Gbeta gamma Subunits and Phospholipase C-gamma 1 in Intestinal Epithelial Cells. *Journal of Biological Chemistry*, *275*(13), 9849–9853. doi:10.1074/jbc.275.13.9849

Tzingounis, A. V., von Zastrow, M., & Yudowski, G. A. (2010). Beta-blocker drugs mediate calcium signaling in native central nervous system neurons by beta-arrestin-biased agonism. *Proceedings of the National Academy of Sciences*, *107*(49), 21028–33. doi:10.1073/pnas.1004169107

Umapathy, N. S., Zemskov, E. A., Gonzales, J., Gorshkov, B. A., Sridhar, S., Chakraborty, T., … Verin, A. D. (2010). Extracellular beta-nicotinamide adenine dinucleotide (beta-NAD) promotes the endothelial cell barrier integrity via PKA- and EPAC1/Rac1-dependent actin cytoskeleton rearrangement. *Journal of cellular physiology*, *223*(1), 215–23. doi:10.1002/jcp.22029

Wright, J. R., & Dobbs, L. G. (1991). Regulation of pulmonary surfactant secretion and clearance. *Annual review of physiology*, *53*, 395–414. doi:10.1146/annurev.ph.53.030191.002143

Yang, S., Cheek, D. J., Westfall, D. P., & Buxton, I. L. (1994). Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circulation Research*, *74*(3), 401–407. doi:10.1161/01.RES.74.3.401

Zhang, W., Yano, N., Deng, M., Mao, Q., Shaw, S. K., & Tseng, Y.-T. (2011). β-Adrenergic receptor-PI3K signaling crosstalk in mouse heart: elucidation of immediate downstream signaling cascades. *PloS one*, *6*(10), e26581. doi:10.1371/journal.pone.0026581

Zink, S., Rösen, P., Sackmann, B., & Lemoine, H. (1993). Regulation of endothelial permeability by beta-adrenoceptor agonists: contribution of beta 1- and beta 2-adrenoceptors. *Biochimica et biophysica acta*, *1178*(3), 286–98.

FIGURES

FIGURE 1

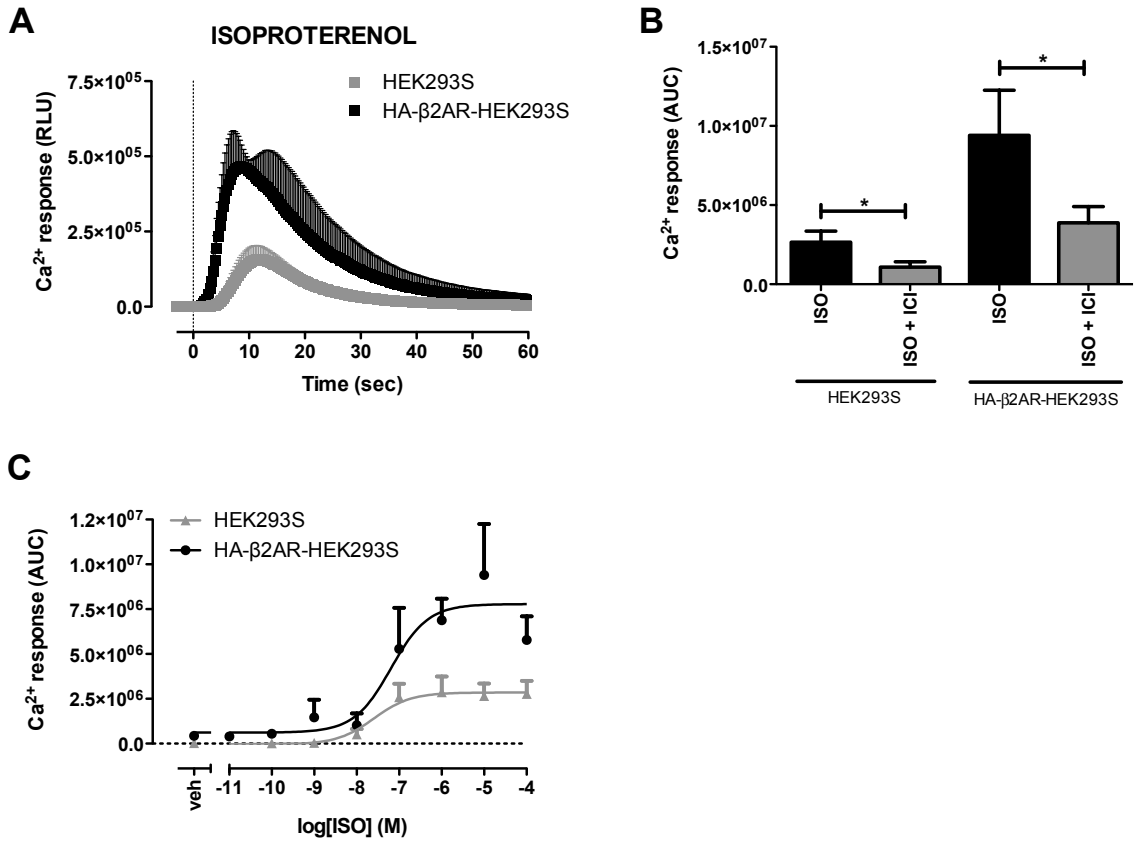


Figure 1 – Isoproterenol stimulates an increase in intracellular Ca^{2+} via the β_2AR. (A) Injection of isoproterenol (ISO; 10 μM) at t=0 increases intracellular Ca^{2+} in HEK293S and HA-β_2AR-HEK293S cells, as demonstrated by increases in obelin luminescence (RLU). (B) The ISO-promoted Ca^{2+} response was blocked in cells pre-treated with the β_2AR antagonist, ICI 118,551 (100 nM; 1 h pre-treatment). (C) The ISO-promoted Ca^{2+} response was concentration-dependent in both HEK293S and HA-β_2AR-HEK293S cells with pEC_{50} values of 7.66±0.16 and 7.12±0.24, respectively. Data are mean ± s.e.m. of 3-4 independent experiments with repeats in triplicate. Area under the curve (AUC) data in column graphs were analyzed by one-tailed paired student's t-test, where $p<0.05$ (*) was considered significant.

Figure 2

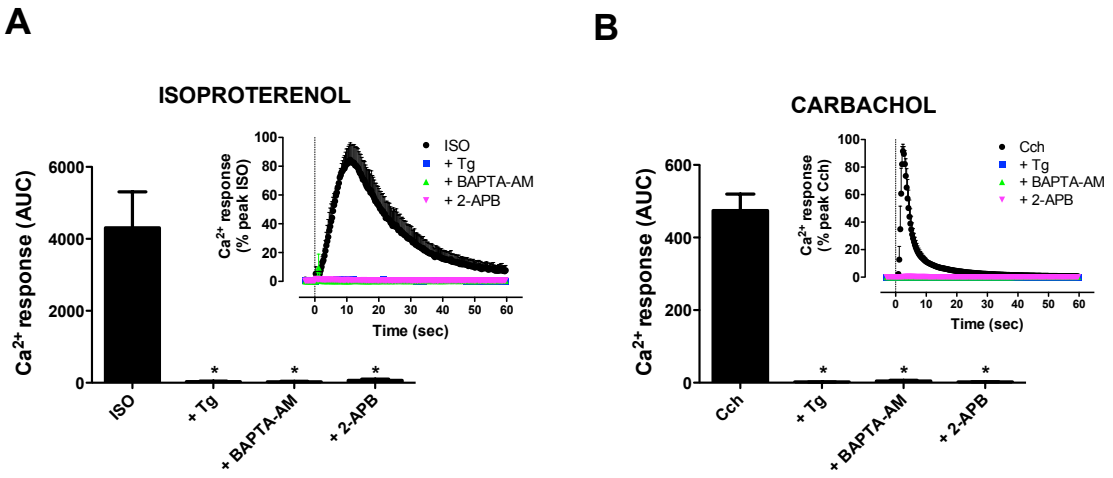


Figure 2 – Ca^{2+} is mobilized from the intracellular stores upon stimulation with ISO or carbachol. Pre-treatment of HA-β_2AR-HEK293S cells with thapsigargin (Tg; 5 µM, 1 h), BAPTA-AM (20 µM, 1 h), or 2-aminoethoxydiphenyl borate (2-APB; 200 µM, 1 h) completely blocked the Ca^{2+} response following injection of (A) ISO (10 µM) or (B) carbachol (Cch, 100 µM). Data are mean ± s.e.m. of 3-6 independent experiments with repeats in triplicate. Area under the curve (AUC) data in column graphs were analyzed by one-way ANOVA, with a Dunnett's multiple comparison *post-hoc* test, where $p<0.05$ (*) was considered significant.

Figure 3

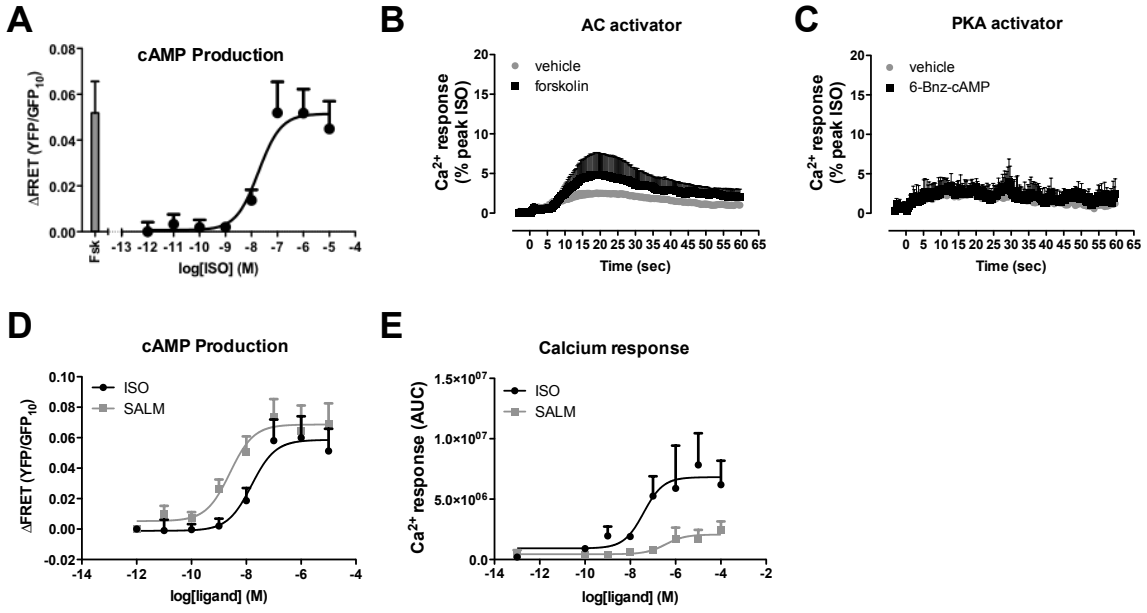


Figure 3 – ISO-promoted Ca^{2+} mobilization is independent of cAMP production. (A,B) Forskolin (100 μM, 30 min) activates cAMP production in HA-β_2AR-HEK293S cells to a similar extent as ISO (10 μM, 30 min) (A), but stimulates only a very weak Ca^{2+} response (B). (C) The PKA activator, 6-Bnz-cAMP (500 μM) does not stimulate the mobilization of Ca^{2+}. (D,E) ISO and salmeterol (SALM, 30 min) promote similar cAMP production (D); however, SALM stimulates far less Ca^{2+} mobilization (E). Data are mean ± s.e.m of 3-6 independent experiments with repeats in duplicate or triplicate.

Figure 4

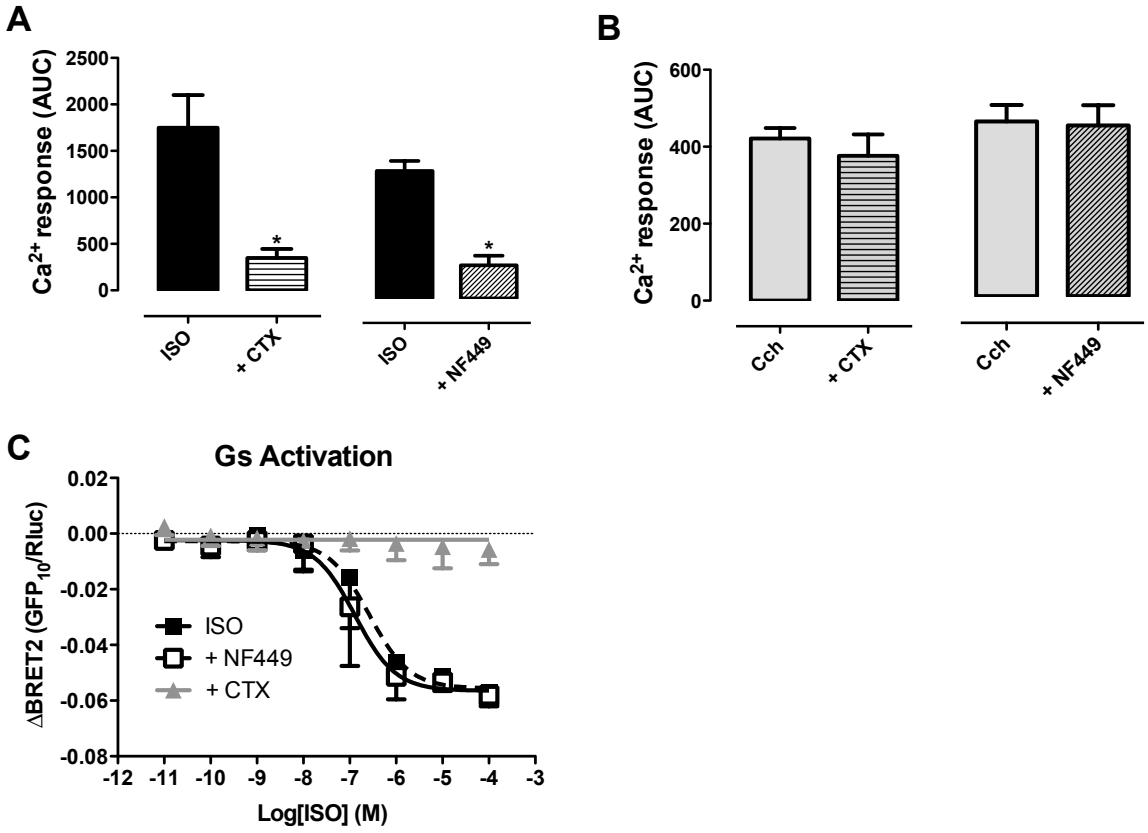


Figure 4 – ISO-promoted Ca^{2+} mobilization is Gs-dependent. (A,B) Chronic pre-treatment of HA-β_2AR-HEK293S cells with cholera toxin (CTX) (200 ng/ml; 24 h) or acute pre-treatment with NF449 (10 μM; 1 h) significantly inhibited ISO-promoted (10 μM) Ca^{2+} mobilization (A), but had no effect on the Cch-promoted (100 μM) Ca^{2+} response (B). (C) ISO promotes a concentration-dependent decrease in the BRET observed between Gs-67-RlucII and GFP10-Gγ1 indicating Gs activation. Pre-treatment with CTX, but not NF449, completely inhibits ISO-promoted Gs activation. Data are mean ± s.e.m. of 3-6 independent experiments with repeats in duplicate or triplicate. Area under the curve (AUC) data presented in column graphs were analyzed by two-tailed paired t-tests, where $p<0.05$ (*) was considered significant.

Figure 5

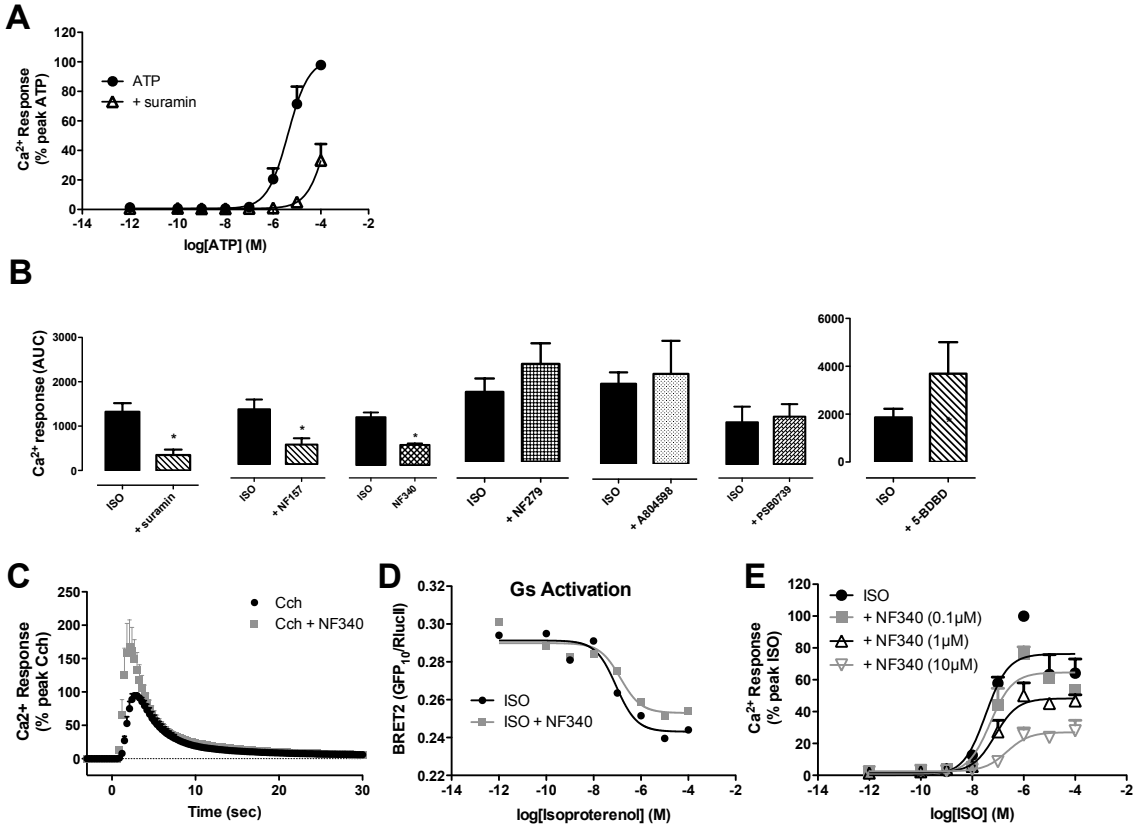


Figure 5 – ISO-promoted Ca^{2+} mobilization is inhibited upon P2Y11 purinergic receptor blockade. (A) The purinergic receptor agonist, adenosine triphosphate (ATP, 100 μM) increases intracellular Ca^{2+} in HA-β_2AR-HEK293S cells in a concentration-dependent manner. This response is competitively inhibited in cells pre-treated with the pan-purinergic antagonist suramin (500 μM; 1 h). (B) ISO-promoted (10μM) Ca^{2+} mobilization was inhibited in cells pre-treated for 1 h with suramin, NF157 (10 μM) and NF340 (10 μM), but not with NF279 (1 μM), A804598 (1 μM), PSB0739 (1 μM) or 5-BDBD (10 μM). (C) NF340 does not block the Cch-promoted (100 μM) Ca^{2+} response. (D) NF340 does not block ISO-stimulated Gs activation. (E) The concentration-dependent increase in intracellular Ca^{2+} promoted by ISO is inhibited upon pre-treatment with NF340 in a non-competitive manner. Data are mean ± s.e.m. of 3-4 independent experiments with repeats in triplicate. Area under the curve (AUC) data presented in column graphs were analyzed by two-tailed unpaired t-tests, where $p<0.05$ (*) was considered significant.

Figure 6

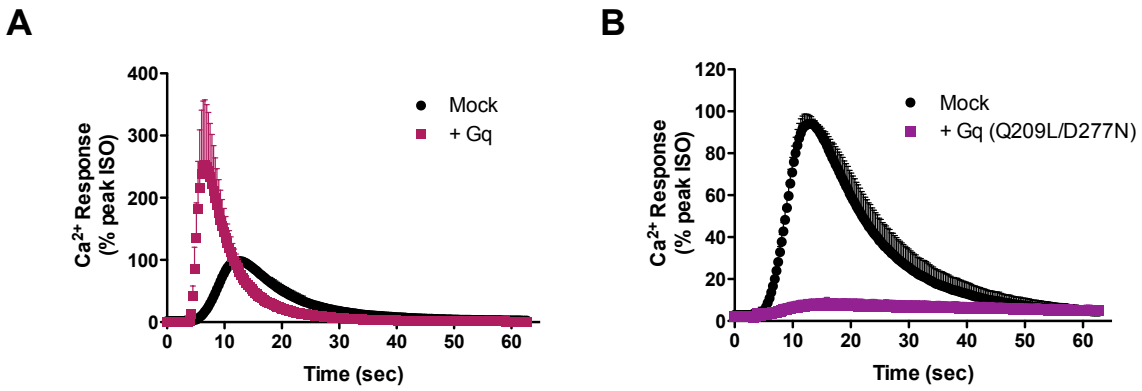


Figure 6 – ISO-promoted Ca^{2+} mobilization is modulated by wild-type or dominant negative Gq expression. (A,B) Overexpression of wild-type Gq in HA-β_2AR-HEK293S cells potentiates ISO-promoted (10 μM) Ca^{2+} mobilization (A), while overexpression of a dominant negative mutant of Gq (Q209L/D277N) blocks this response (B). Data are mean ± s.e.m. of 4-5 independent experiments with repeats in triplicate.

Figure 7

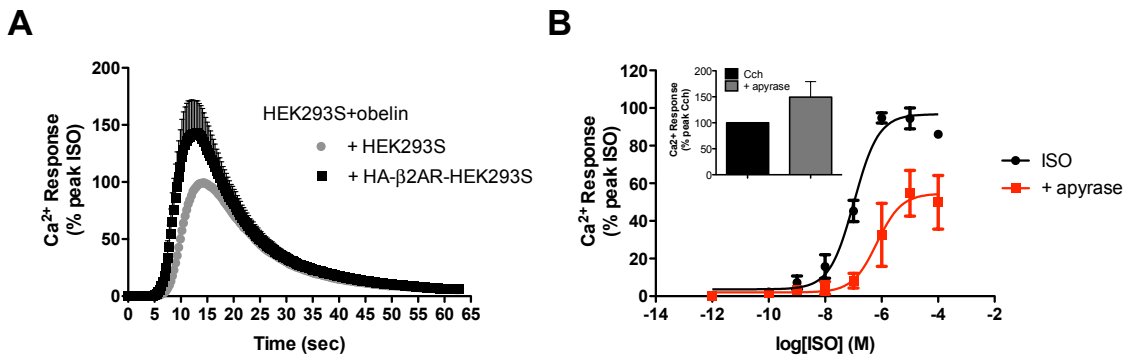
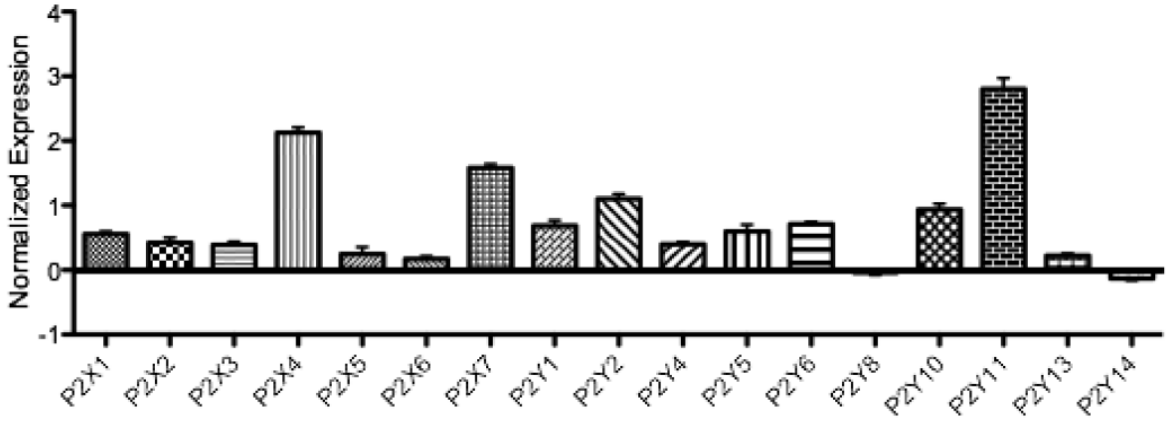


Figure 7 – ISO promoted the extracellular release of ATP. (A) Parental HEK293S cells were transfected with obelin-mCherry and co-cultured with HEK293S or HA-β_2AR-HEK293S cells not expressing the biosensor. Stimulation with ISO (10 µM) promotes a greater increase in intracellular Ca^{2+} in HEK293S-obelin cells that were co-cultured with HA-β_2AR-HEK293S cells compared to the HEK293S parental cells. (C) ISO-promoted Ca^{2+} mobilization is inhibited in cells that were pre-treated with apyrase (1 ui/ml; 1 h), however, the response to Cch (100 µM) was unaffected (inset). Data are mean ± s.e.m. of 3 independent experiments with repeats in triplicate.

Supplementary Figure 1



Supplemental Figure 1 – mRNA expression of P2X and P2Y purinergic receptor subtypes in HA-β_2AR-HEK293S cells. Data are expressed as fold-over the mean background signal of the assay.

ARTICLE 3

Chemical Systems Biology Approach for the Functional Classification of Ligands Targeting the β_2 adrenergic receptor

Wayne Stallaert, Jonas F. Dorn & Michel Bouvier, 2013.
Finalized manuscript for submission

Context/Objectives:

This article represents a significant technical and conceptual extension of the work presented in Article 1 (page 63) on the use of cellular impedance to screen for functionally selective compounds at GPCRs. In this study, impedance screening was combined with the use of pathway-selective inhibitors to reveal both differences in the overall cellular response, as well as the contribution of six β_2AR effectors to this response, among a library of compounds targeting the β_2AR. In addition, this novel approach was used to characterize and classify an uncharacterized library of β_2AR compounds, revealing compound classes with functional properties distinct from those currently used in the laboratory and clinic.

Impedance signatures were obtained for 62 compounds known to target the β_2AR. These included 21 well characterized compounds with recognized signalling biases at the receptor ("Reference Set"), as well as 41 compounds that act selectively at the receptor but whose signalling activity has yet to be characterized ("Test Set"). For each of the compounds, impedance responses were obtained following pre-treatment with six pathway-selective inhibitors covering various arms of the known β_2AR signalling repertoire: Gs-dependent signalling (cholera toxin, CTX); Gi-dependent signalling (pertussis toxin; PTX); cAMP production (SQ22536); ERK1/2 activation (U0126); Akt activation (PI-103); and NF-κB signalling (Ro 106-9920). We then developed a novel computational approach to (1) quantify the sensitivity of each compound impedance signature to each pathway inhibitor, (2) visualize the relative contributions of pathways to a compound's impedance response, and (3) cluster compounds by the impedance profiles

obtained. Prototypical compounds from each of the predicted compound classes were subsequently assessed for their ability to modulate human induced pluripotent stem cell (iPSC)-derived cardiomyocyte contraction.

The combined use of cellular impedance and pathway inhibitors provided greater resolution in detecting functional differences among the compounds assessed. Compounds eliciting similar impedance signatures could be further distinguished by detecting differences in relative pathway contributions to the overall cellular response. Using this impedance profiling approach across the entire compound library produced eleven distinct compound classes, seven containing both Reference and Test Set ligands and four potentially novel compound classes containing only Test Set ligands. Prototypical compounds from each of the compounds classes were found to elicit distinct effects on human cardiomyocyte contractility, validating the ability of impedance profiling to detect signalling biases among a compound library that translate into distinct functional properties in a relevant physiological context.

Combining cellular impedance and pathway inhibitors provides an efficient and accurate means to assess ligand functional selectivity at GPCRs and can functionally discriminate among compounds with distinct functional properties *in vivo*.

My contribution as first author included the conception and execution of all of the experiments conducted except for the cardiomyocyte contractility assay. I also participated in much of the analysis, with the exception of the computational approach developed to quantify, visualize and cluster impedance profiles. I wrote the first draft of the manuscript and participated greatly in its subsequent revision.

Chemical Systems Biology Approach for the Functional Classification of Ligands Targeting the β_2 adrenergic receptor

Running title: Impedance profiling of β_2AR ligands

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Key words: biosensors, drug discovery, G protein-coupled receptors, cellular impedance, cardiomyocytes

ABSTRACT

The discovery that drugs targeting a single G protein-coupled receptor (GPCR) can differentially modulate distinct subsets of the signalling repertoire engaged by the receptor ("functional selectivity") has created a formidable and exciting challenge for drug discovery at this important class of therapeutic targets. In the present study, we demonstrate that a label-free assay based on cellular impedance in combination with pathway-selective inhibitors covering the β_2 adrenergic receptor (β_2AR) signalling repertoire allows the clustering of ligands into distinct compound classes that reflect differences in their overall signalling profiles. This chemical systems biology approach allowed us to classify a Reference Set of 21 well-characterized β_2AR ligands, as well as a library of 41 uncharacterized β_2AR ligands based on their impedance profiles, leading to the identification of potentially novel compound classes. These putative compound classes were subsequently validated to elicit distinct effects on human cardiomyocyte contractility. These data demonstrate a novel use of such label-free techniques as systems-based approaches to study GPCR signalling networks and ligand functional selectivity.

INTRODUCTION

Ligand functional selectivity presents exciting opportunities for drug discovery at GPCRs. This phenomenon, also referred to as biased signalling, is characterized by the ability of compounds to differentially modulate subsets of pathways coupled to a single receptor species (Kenakin & Christopoulos, 2012; Stallaert, Christopoulos, & Bouvier, 2011; Urban et al., 2007). Pathways providing therapeutic benefit in a pathophysiological context can be selectively targeted without modulating unrelated, unnecessary pathways that contribute to the development of adverse effects or tolerance. However, fully characterizing the overall signalling profiles of ligands and their impact on overall cellular biology has remained a formidable challenge.

Label-free techniques, including both cellular impedance (Peters & Scott, 2009; Stallaert, Dorn, van der Westhuizen, Audet, & Bouvier, 2012; Yu et al., 2006) and dynamic mass redistribution (Schröder et al., 2010), represent a novel and attractive approach to assess GPCR signalling networks. These techniques detect changes in higher order cellular events following receptor activation, such as changes in morphology, adhesion and the redistribution of macromolecules, providing an integrative measure of the cellular response to drug treatment. Any signalling events converging on these cellular responses will be represented in the measured impedance response in real-time and in living cells to provide a "signature" of ligand activity by integrating the overall signalling profile of a compound at a given receptor into a single readout (Stallaert et al., 2012). The contribution of individual signalling events to these integrative readouts can easily be dissected through the use of pharmacological or genetic manipulations (Schröder et al., 2010; Stallaert et al., 2012).

In the current study, we present a novel approach to assess ligand functional selectivity at the β_2 adrenergic receptor (β_2AR) using cellular impedance. We have screened a total of 62 compounds targeting the β_2AR and obtained impedance signatures representative of their overall signalling profiles at the receptor. We have combined this impedance screening with the use of pathway-selective inhibitors for a variety of signalling events in the β_2AR repertoire to assess the contribution of individual pathways to impedance responses. A

novel computational approach was developed and applied to these data which allowed us to (1) calculate the contribution of each of these pathways to ligand impedance signatures, (2) visualize the impedance profiles of ligands and (3) cluster compounds into distinct functional classes. Twenty-one of these compounds were well-characterized ligands for the β_2AR, reflecting the diverse signalling bias well documented for ligands targeting the receptor (Galandrin & Bouvier, 2006; Liu, Horst, Katritch, Stevens, & Wüthrich, 2012; Patel, Noor, & Rockman, 2010; Reiner, Ambrosio, Hoffmann, & Lohse, 2010), including many drugs currently used in the clinic. Using these as a Reference Set, we subsequently assessed a library of 41 uncharacterized compounds to investigate the ability of this technique to be predictive of a ligand's signalling profile. Our analysis revealed that while many of these uncharacterized compounds possess high functional similarity to the compounds in the Reference Set, several compounds were found to exhibit distinct impedance profiles, suggestive of novel functional classes of ligands targeting the β_2AR. Compound classes were subsequently found to elicit distinct effects on human cardiomyocyte contractility, providing further functional validation of the clustering predicted by impedance profiling in a relevant physiological context.

RESULTS

Pharmacologically distinct β_2AR ligands generate diverse impedance signatures

A library of β_2AR compounds encompassing the full pharmacological space of the receptor was selected as a Reference Set to validate the combined use of impedance and pathway-selective inhibitors to screen ligand signalling profiles (**Supplementary Table 1**). This β_2AR compound library included many of the ligands classified by a previous impedance screen into 5 distinct functional groups (Stallaert et al 2012). A wide variety of impedance signatures was obtained in HEK293S cells heterologously expressing an N-terminally tagged human β_2AR (HA-β_2AR-HEK293S cells), with each compound in the Reference Set eliciting a complex curve consisting of multiple discrete features (**Figure 1, Supplementary Figure 1**). Many of the signatures immediately dip to negative values, varying between a more transient (2-3 minutes for isoproterenol (ISO), epinephrine (EPI) and fenoterol (FEN)) or sustained (5-8 minutes for alprenolol (ALP), pindolol (PIN), bucindolol (BUC) and labetalol (LAB)) negative phase, before ascending to positive values (**Figure 1b,d**). Furthermore, some compounds exhibit two components in this ascending phase; first with a rapid rise followed by an abrupt change to a more gradual slope (**Figure 1a,c**), while others (propranolol (PRO), timolol (TIM)) only elicit a single ascending phase (**Figure 1e**). Most compound signatures reach a subsequent maximum positive response between 20 and 30 minutes, the amplitude of which varying greatly among compounds (**Figure 1f**). Other compounds (ICI118,551 (ICI), metoprolol (MET), nadolol (NAD)) exhibit predominantly negative responses, reaching a minimum ranging between 20 and 40 minutes (**Figure 1g**). Close analysis of these individual features reveals that each can contribute independently to the overall impedance response. Ligands may exhibit similar negative phases but reach distinct maxima (EPI vs. FEN) (**Figure 1a,b**), while others differ in the amplitude and duration of the early negative phase, yet reach similar maxima (PRO vs. TIM) (**Figure 1e**). Others yet differ in the relative contributions of the rapid and slow ascending phases despite rising to a similar maximum impedance response (salbutamol (SALB) vs. dichloroisoproterenol (DIC)) (**Figure 1h-j**). Since earlier work has established that these individual phases of the impedance response correlate with the activation of

distinct signalling events (Schröder et al., 2010; Stallaert et al., 2012), these data suggest that differences in the signalling profiles of compounds may be represented by variations in the independent features of their impedance signatures.

Combining impedance with pathway inhibitors reveals additional distinctions among ligands

To further explore the contribution of individual signalling events to the impedance signatures obtained, cells were pre-treated cells with 6 pathway-selective inhibitors targeting distinct arms of the β_2AR signalling repertoire. The contribution of Gs and Gi signalling were assessed by chronic pre-treatment with cholera toxin (CTX) or pertussis toxin (PTX), respectively. Downstream signalling responses, such as the production of cAMP (AC inhibitor, SQ22536), activation of ERK1/2 (MEK inhibitor, U0126) and Akt (PI3K inhibitor, PI-103) pathways, and the contribution of the β-arrestin-dependent modulation of NF-κB signalling (IκB ubiquitination inhibitor, Ro 106-9920) were also assessed by acute pharmacological inhibition (**Supplementary Figure 2a**). With additional information about if and how a particular signalling event contributes to a compound's impedance response, ligands that otherwise exhibit very similar signatures can be further differentiated. For example, SALB and salmeterol (SALM) produce very similar impedance signatures and have been shown to cluster together into a single functional class in a previous impedance screen (Stallaert et al 2012). These ligands exhibit identical early phases and only minor differences in maximum response (**Figure 2a,b**); however, when we examine how individual signalling events contribute to their impedance responses, key differences are observed that functionally differentiate these compounds. Gi-dependent signalling, for example, contributes to a much larger proportion of the SALB impedance response (**Figure 2c,d**). We also observe differences in the contribution of NF-κB signalling (**Figure 2e,f**), as Ro 106-9920 pre-treatment leads to a more rapid decline from the maximum SALB response compared to SALM. A more striking example of how pathway inhibitors differentially affect impedance responses is demonstrated by the comparison between ICI, NAD and MET (**Figure 1g, Figure 3**). While each of these responses can be almost entirely inhibited upon CTX pre-treatment to abolish Gs signalling (**Figure 3a-c**), the contribution of NF-κB signalling, on the other hand, differs substantially among the

ligands. Inhibition of this pathway leads to greater negative responses for ICI and NAD, while the MET impedance response becomes more positive (**Figure 3d-f**). Additional differences are revealed when we examine the impact of ERK1/2 signalling on these responses. Inhibition of this pathway has very little impact on the ICI impedance signature, yet causes the MET response to become more positive and completely inverts the NAD impedance response to an entirely positive response (**Figure 3g-i**). Thus, these two examples demonstrate that although ligands may elicit very similar impedance signatures, assessing the contribution of individual pathways to each impedance response can reveal functional differences among ligands that might otherwise go undetected.

Conserved temporal phases in β_2AR impedance response

Ligand impedance signatures exhibit important distinctions in both the early and transient phases as well as in the later, more prolonged phases. In order to further analyze these responses and balance the influence of each in the analysis, we expressed time on a log-scale to effectively expand the early events and compress later events. Log transformation of the impedance signatures revealed a very interesting property of the responses that was not apparent when time was expressed linearly. When expressed on a log-time scale, compounds produce wave-like impedance responses (**Figure 4a**) with conserved patterns of oscillations at similar time regimes (**Figure 4b**). This transformation highlights at least four distinct phases in the β_2AR-promoted impedance response corresponding to immediate (≈ 1-2 min), early (≈ 7-10 min), intermediate (≈ 20-25 min) and later (≈ 30-40 min) phases, whereby ligands are distinguished by the presence or absence of each, and by the magnitude, direction and timing of the phases. Furthermore, we observed that the similarity among ligands is highest in both direction and timing in the earlier phases, while later phases exhibit more heterogeneity.

Classification of ligands using impedance and pathway inhibitors

Given the additional information provided when combining impedance screening with pathway inhibitors (**Figures 2 and 3**), our goal was to develop a quantitative measure of dissimilarity among ligand impedance profiles that could cluster compounds into distinct

functional classes, taking into account the effect of pathway inhibitors on each compound signature. We hypothesized that a good measure of dissimilarity would allow the clustering of ligands into known functional classes and might reveal subgroups within those classes. To this end, we chose a three-step approach to: (1) describe impedance signatures accurately using a minimal number of parameters, (2) combine the effects of pathway inhibitors on ligand impedance signatures into a set of simple descriptors, and (3) choose a dissimilarity function between these descriptors that would allow clustering of compounds into functional classes.

In order to describe impedance signatures using a minimal number of parameters, we defined a set of basic curves, called eigenshapes, with which we could reproduce any of the impedance signatures through linear combination (i.e. by multiplying each eigenshape by a factor and summing them). We derived the set of eigenshapes using principal component analysis on resampled impedance signatures (see *Methods*). Analysis revealed that linear combinations of a set of seven distinct eigenshapes (**Figure 4c**) could explain >97.5% of the variance across the entire data set (**Figure 4d**) and provide an accurate approximation of each impedance response (**Figure 4e**). Thus, each of the impedance responses can now be described by a mathematical function containing seven discrete variables (i.e. the factors by which the seven eigenshapes were multiplied to reproduce the full impedance response of a given ligand).

Next we sought to create a specific set of descriptors for each ligand that reflect the sensitivity of its impedance signature to each pathway inhibitor. Each condition (control or pathway inhibitor pre-treated) for a given ligand can be expressed as a vector in seven-dimensional space, given by the seven eigenshape factors that are used to reproduce the ligand's impedance response. As a measure of the difference between the control and each of the pathway inhibitor pre-treated impedance responses, we used a function of the cosine of the angle between the vectors for each pairwise comparison. Consequently, we obtain a set of descriptors, one for each of the pathway inhibitor conditions, that represents the overall "impedance profile" of a ligand reflecting the relative contribution of each pathway to its impedance response. To visualize this set of descriptors, we constructed "star plots"

representing the sensitivity of each of the Reference Set ligands to the six pathway inhibitors (**Figure 4f**). In these plots, the distance between the center of the star and each corner represents the sensitivity of an impedance response to a given pathway inhibitor.

Examination of the star plots generated for the Reference Set suggests vast differences in the impedance profiles of ligands. We next sought to obtain a single quantitative measure to characterize this dissimilarity among ligands, which would allow for the clustering of compounds into distinct functional classes. We proposed that a modified cosine distance is a sensitive measure of dissimilarity between impedance profiles. To test this proposition, we used this dissimilarity measure to hierarchically cluster the ligands by their star plots, and asked whether the clustering would produce meaningful groups. Indeed, the dendrogram exhibits three major branches, representing ligands traditionally classified as (A) strong agonists (B) partial agonists and (C) ligands often considered neutral antagonists or inverse agonists (clinically referred to as "beta blockers"); however, within each of these branches are multiple subgroups that could represent additional signalling distinctions and biases among the compounds. The accordance between the clustering of ligands based on their impedance profiles and the biological function previously attributed to these compounds further validates the use of this approach to characterize ligand functional selectivity.

Combining impedance screening with pathway inhibitors to predict ligand signalling biases

This approach to ligand classification was then assessed for its capacity to predict functional differences among an uncharacterized β_2AR compound library. Forty-one compounds ("Test Set") were screened using impedance in combination with the same six pathway inhibitors (**Supplementary Figure 2b**). Analysis revealed that seven eigenshapes were once again sufficient to explain >97.5% of the variance for all impedance signatures (**Figure 4d**) and log-time transformation produced wave-like impedance responses with local oscillations at similar time regimes as the Reference Set (**Figure 4b**). Nearly all of the Test Set compounds were found to β_2AR-specific since their impedance responses could be completely inhibited upon pre-treatment with the β_2AR antagonist carvedilol, which had no effect on the impedance response generated by the muscarinic receptor agonist carbachol

(**Supplementary Figures 3 and 4**). The impedance responses of BI754 and TX10 were not completely inhibited by carvedilol, indicating that there might be a non-β_2AR component for these ligands. Star plots were obtained for all of the Test Set ligands and a dendrogram consisting of the entire compound library was determined. When the Reference and Test Sets were analyzed together, we obtained a dendrogram (**Figure 5a**) with a far greater number of possible compound classes than when the Reference Set alone is considered. Interestingly, some of the Test Set ligands cluster into compound classes that are distinct from those generated by the Reference Set (Groups 2, 5, 8 and 9), suggesting that these ligands might represent novel compound classes. To examine the mechanistic basis for the clustering observed and reveal potential pathway biases among ligands, we quantitatively compared the individual pathway contributions that produce the star plots for each ligand (**Figure 5b**). In general, the pathways that contribute the most to distinguishing ligand classes are also the most upstream targets of inhibitors, Gs (CTX-treated) and Gi (PTX-treated), while the other downstream pathways (NF-κB, Akt, cAMP and ERK1/2) further differentiate compound classes with similar Gs/Gi profiles.

Functional distinctions in cardiomyocytes

To determine if the clustering obtained through impedance profiling translates into distinct functional responses in human cells, prototypical compounds representing each of the putative compound classes were assessed for their effects on human induced pluripotent stem (iPS) cell-derived cardiomyocyte contractility (**Figure 6**). After one hour of compound treatment, we measured changes in several parameters of the contractile response including beat rate, amplitude and shape. Comparing the effects of compounds on beat rate and amplitude (**Figure 6b**), we observe a variety of effects that functional differentiate many of the compound groups. Great differences in the extent to which compounds modulate the frequency of contraction are observed, ranging from little to no effect (ICI), decreases (LY26 and PRO) or varying extents of increases in contractile frequency (LY09 ≥ BI07 ≥ ISO ≥ TX177 > BI80 > SALM > LY11 > SALB). We also observe differences in the ability of compounds to induce changes in the amplitude of the response. While some compounds induce a decrease in amplitude (LY09, BI80, BI07, TX177), others increase the

amplitude (LY26) or have no effect (PRO, ICI, SALB, LY11, SALM, ISO). These properties alone are not sufficient to differentiate all of the groups, as we observe that TX177 (Group 2), BI07 (Group 6), LY09 (Group 8) and BI80 (Group 11) elicit very similar effects on beat rate and amplitude despite belonging to distinct compound classes in the impedance profiling. However, when we consider the effect of compounds on the shape of the contractile response by comparing quantitative changes in the positive and negative peaks (**Figure 6c**), we are able to further differentiate the compounds tested. For example, LY09 and BI07 induce very distinct changes in the shape of the contractile response (LY09 greatly increases the negative peak and decreases the positive peak, while BI07 decreases both peaks). However, we were still not able to differentiate TX177 and BI80 by their effects on beat shape. Together these three parameters of the contractile response in cardiomyocytes are sufficient to distinguish 9 out of 11 of the compound classes, demonstrating that the classification of ligands based on their impedance profiles provides an accurate representation of their functional properties in a human physiological context.

DISCUSSION

In recent years, ligand functional selectivity has become recognized as an important aspect of drug discovery at GPCRs. Techniques to observe, characterize and exploit this phenomenon are of great interest in both academic laboratories as well as in the pharmaceutical industry. The current study reveals additional biological insight into this exciting new paradigm of GPCR pharmacology and provides an alternative and complementary approach to other techniques that are currently being used to study it.

Most attempts to characterize ligand functional selectivity and calculate signalling bias have relied on the direct measurement of discrete signalling outcomes (e.g. production of second messengers, activation of kinases, etc.). While these approaches have found previous success in identifying signalling distinctions among compound libraries, the biological significance of these biases remains subject to further study. Impedance profiling, on the other hand, provides insight into the biological relevance of a given signalling bias by assessing its importance in the overall cellular response to ligand treatment. Given the intricate and integrative nature of cellular signalling networks, the activation state of a given pathway may lead to distinct cellular outcomes depending on the status of the rest of the network. Therefore, the signalling profiles suggested by impedance profiling (i.e. star plots) may or may not directly correspond to the activation state of each of the pathways, if they were assayed in isolation. Instead, these profiles represent the "importance" of each pathway in the overall cellular response, which may in fact provide a more accurate assessment of the functional differences among ligands *in vivo*.

Similarly, to calculate the contribution of a given pathway to a ligand impedance response, our analysis places a greater focus on qualitative rather than quantitative changes to the overall signature. For example, if pre-treatment with one pathway inhibitor fundamentally changes the overall shape of the impedance response to a ligand, our method would ascribe a larger contribution to that pathway than if an inhibitor merely changed the amplitude of an impedance response with no change to the overall shape. In doing so, we attempted to emphasize the key signalling components that fundamentally determine the overall cellular response, rather than those that simply "fine-tune" the response. We reasoned that this

type of analysis would allow us to detect and quantify the most important differences among compound signalling profiles. We observed that the effectors that contribute the most in distinguishing compound classes are Gs and Gi. Given that these effectors are the most upstream signalling components of GPCRs, it is not surprising that they contribute most significantly to ligand impedance responses, since inhibiting these G proteins directly will also affect downstream pathway components such as cAMP, ERK1/2 and Akt, as well as additional G protein-dependent signalling events not directly assessed in the current study.

We subsequently confirmed that the compound classes proposed by impedance profiling accurately predict functional distinctions among ligands in a highly relevant physiological context, as ligands from different groups elicit distinct effects on human cardiomyocyte contractility. We were able to distinguish 9 out of 11 groups based on their ability to modulate the contractile response. Only groups 2 and 11 were not found to exhibit functional differences in cardiomyocytes; however, it is possible that the signalling that differentiates these groups might not contribute to the contractile response, and thus would not be detectable using this assay alone. Assessment of ligand activity on other β_2AR physiological outcomes such as smooth muscle tone (Guimaraes & Moura, 2001) or migration (Johnson, Webb, Newman, & Wang, 2006) might instead reveal the functional differences between these compound classes.

Log-time transformation of impedance signature revealed a clear periodicity in the cellular response to ligand treatment. The conservation of similar time regimes across the compound library may indicate the temporal separation of distinct biological phenomenon. Given the coincidence of these time points with the kinetics of known signalling events elicited upon β_2AR activation, it is tempting to postulate that these phases of the impedance response might correspond to the maximal cellular effects of (1) immediate, membrane delimited reorganization of macromolecules (≈ 1-2 minutes), (2) production of 2nd messengers such as cAMP (≈ 7-10 minutes), (3) full activation of kinases cascades and their downstream targets (≈ 20-25 minutes) and (4) modulation of gene transcription (≈ 30-40 minutes). The observation that these oscillations at conserved time regimes are more

heterogeneous across the compound library for the later events suggests that small differences in the early signalling events (e.g. G protein activation) are propagated to produce larger changes later in the biological response to receptor stimulation.

There currently exist several challenges in exploiting functional selectivity for therapeutic benefit. First, it can be an expensive endeavor in both time and resources to screen the signalling profiles of a large compound library. Individual assays must be developed, optimized and performed for each of the signalling events to be measured. These assays are often performed in different experimental conditions, which may influence the pharmacology of the compound towards each signalling event due to differences in the sensitivity of the assays, or whether the endpoint is measure in live cells or lysate, for example. Differences in the nature of the signalling events being assessed may also convolute the results. The efficacy of a compound could be found to differ between two pathways; however, these differences may simply reflect differences in the level of amplification of each pathway. Furthermore, this approach relies on possessing the knowledge of most, if not all, of the receptor's signalling repertoire. Important pathways contributing to the overall cellular response may not be assessed and thus important differences in the signalling profiles of ligands could be missed. Not considering the full spectrum of signalling events modulated by ligand would significantly reduce the predictive potential of such an approach on the physiological or therapeutic effects of drugs, including the potential for adverse effects.

Combining impedance screening with pathway inhibitors addresses several of these shortcomings; allowing potential ligand signalling biases to be identified using a single assay format, significantly decreasing the time and resources required to assess functional selectivity among a compound library and eliminating potential confounding factors associated with different assay formats. In the current study we have screened six pathway inhibitors selected to provide coverage over much of the known β_2AR signalling repertoire; however, future screens could involve the inclusion of inhibitors of pathways not previously identified to be coupled to the receptor. Yet since the impedance signature represents the full signalling spectrum of a given ligand, even when the contribution of an

unknown pathway is not directly assessed with an inhibitor, their influence remains a component of the overall impedance response.

In addition, the generation of star plots representing the impedance profiles of a compound library provides a highly effective means of visualizing ligand functional selectivity. Previous attempts to visualize this signalling diversity have been limited to either Cartesian representation of ligand efficacies (Galandrin & Bouvier, 2006) or "bias plots" comparing the pharmacological activity of ligands towards two pathways (Rajagopal et al., 2011). While highly effective at incorporating multiple pharmacological properties into the analysis (i.e. efficacy, potency, receptor affinity), bias plots can only visualize potential biased signalling between two pathways at a time. In addition, calculating signalling bias has thus far been dependent on the use of a reference ligand, often the endogenous agonist for a given receptor, under the assumption that this ligand is itself unbiased. The generation of star plots from impedance profiling data provides a standalone representation of ligand signalling profiles by a simple geometric shape extending into far greater dimensions and with no need of a reference compound. This visualization method should provide researchers with a highly effective tool to efficiently identify signalling bias among a large library of compounds.

The combined use of impedance screening and pathway inhibitors could be valuable addition to future drug discovery campaigns. After obtaining a set of hits in a primary screen of the GPCR target using conventional assays, impedance profiling could provide both a validation of the hits obtained and identify functionally distinct classes of compounds among these hits. This approach would be particularly effective when the precise biological determinants of the disease are not firmly established. Multiple lead compounds representing distinct functional classes could be tested in pre-clinical and clinical studies to identify the most effective compound class for treating a given disease. Furthermore, since impedance profiling provides an indication of the pathways modulated by this compound, additional insight into the molecular basis of the illness and/or therapeutic effect may be obtained. Given the label-free nature of this approach, it could also easily be adapted for use in primary or iPS cells to assess ligand activity in tissues in

which the receptor is natively expressed or even in clinically relevant pathological contexts to provide greater predictability of therapeutic efficacy early in the drug discovery process.

METHODS

Materials and reagents

All Reference Set compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA) or R&D Systems (Minneapolis, MN, USA). Test Set compounds were a generous gift from Dr. Brian Kobilka (Stanford University, Palo Alto, CA, USA). SQ22536, U0126, Ro 106-9920 and PI-103 were obtained from R&D Systems (Minneapolis, MN, USA). Cholera toxin and pertussis toxin were obtained from Sigma-Aldrich. Cell culture reagents were from Wisent Incorporated (Montreal, QC, Canada). Other reagents were obtained from Sigma-Aldrich unless stated otherwise.

Cell culture

Impedance profiling was performed in the HA-β_2AR-HEK293S cell line (HEK293S cells stably expressing N-terminal HA-tagged human β_2AR) previously described (Galandrin & Bouvier, 2006) and grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum. Cardiomyocyte contractility studies were performed using human induced pluripotent stem cell-derived cardiomyocytes (iCell cardiomyocytes; catalogue number CMC-100-110-001; Cellular Dynamics International; Madison, WI, USA;). These cardiomyocytes have been reported to possess biochemical and electrophysiological characteristics of normal human heart cells and undergo synchronous contraction (Anson, Kolaja, & Kamp, 2011; Xi et al., 2011). The iCells were thawed and cultured onto E-plate Cardio 96 well plates (ACEA Biosciences, San Diego, CA, USA) according to the protocol provided by Cellular Dynamics International. Briefly, the 6-well plate was coated with 1 ml of 10 ug/ml fibronectin (Sigma-Aldrich) overnight at 4°C. The iCells were thawed and plated in 6-well plates at the density of 750 000 viable and platable cells/well with iCell cardiomyocyte plating medium (iCPM; Cellular Dynamics International) for 48 h, and then replaced with iCell cardiomyocyte maintenance medium (iCMM; Cellular Dynamics International). The maintenance media was refreshed every 2 days. After 7 days cultured in 6-well plates, iCells were trypsinized with 0.1% trypsin (Life Technologies, Grand Island, NY, USA) and transferred onto fibronectin-coated E-plate

Cardio 96 well plates at a density of 30 000 viable cells/well. Compound addition was conducted 7 days after re-plating.

Cellular impedance assay

Cellular impedance assays were performed using the xCELLigence RTCA SP system (ACEA Biosciences). Prior to the experiment, impedance measurements are made with growth medium alone to determine the background cell index in each well of a 96-well E-plate (Roche Applied Science), which is subtracted from the cell index values generated following cell seeding. Cells were plated at a cell density of 20 000 cells/well (or 30 000 cells/well for CTX and PTX pre-treatment conditions) and grown for 16-20 hours before treatment. Cells were pre-treated with inhibitors or vehicle (DMEM) as indicated and subsequently stimulated with ligands. Cell index values (the measurements derived from cellular impedance) were obtained immediately following ligand stimulation every 15 seconds for a total time of at least 90 minutes. Cell index values were normalized across all wells by dividing by the cell index at the time of ligand addition and baseline-corrected by subtracting the cell index obtained in vehicle-treated condition. All data presented in figures represent means of at least three independent experiments.

Cardiomyocyte contractility assay

iCell cardiomyocyte contractility was assessed using the impedance-based xCELLigence RTCA Cardio System (ACEA Biosciences). Maintenance media was refreshed 3 h prior to compound addition. The baseline of the cardiomyocyte beating profile was established 20 minutes before the treatment. Compounds were diluted in the maintenance medium (10x of final concentration) and added to the test wells as indicated. After one hour of compound treatment, cardiomyocyte contraction activity was continuously recorded for 20 seconds. Changes in cardiomyocyte contractility parameters were assessed compared to the pre-treatment beating profile for each condition prior to compound addition.

Data Analysis

Data analysis was performed using RTCA Software 1.2 (ACEA Biosciences), RTCA Cardio Software (ACEA Biosciences), Microsoft Excel (Microsoft, Redmond, WA, USA), GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and MATLAB (The MathWorks, Natick, MA, USA).

Impedance responses were analyzed in a three-step approach: (1) description of impedance responses using a minimal number of parameters, (2) combination of the effects of pathway inhibitors on ligand impedance responses into a set of simple descriptors, and (3) clustering of compounds into functional classes based on a dissimilarity function between these descriptors.

Description of impedance signatures using a minimal number of parameters

Impedance responses were transformed by expressing the time variable on a log scale. We then used principal component analysis (PCA) to describe impedance signatures (Pincus & Theriot, 2007). To make impedance signatures amenable to PCA, we first averaged replicate impedance signatures. As there seemed to be a small effect of differences in cell density on impedance responses, a weighted average of the replicates was performed such that impedance responses with a cell density closest to the average cell density of the entire data set were given highest weights. Specifically, the weight w of signature i is defined as:

$$w_i = e^{\frac{-(m_{CI} - CI_i)}{2\sigma_{CI}}}$$

where m_{CI} is the average initial cell index before treatment (which is a measure of cell density) over all impedance signatures, σ_{CI} the standard deviation of the initial cell index, and CI_i is the initial cell index of signature i. Next, the average impedance responses in time were resampled, since log transformation of the time variable led to more densely packed data points later in the impedance response; thus giving an inordinate weight to later time points in any analysis of the curves. To this end, we defined a set of 21 overlapping triweight kernels K_i that were regularly distributed across the log-time axis:

$$K_i(logT) = \begin{cases} \left(1 - \left(\frac{logT - m}{s}\right)^2\right)^3, \left(\frac{logT - m}{s}\right)^2 \leq 0 \\ 0, \left(\frac{logT - m}{s}\right)^2 > 0 \end{cases} \quad , m_i = \{0.25, 0.5, ..., 4.5\}, s = 0.75$$

which were each multiplied by a factor a_i and summed to describe each signature via the function:

$$f(logT) = \sum a_i K_i(logT)$$

Thus, each impedance response could be described by the 21 factors a_i, calculated on a locally uniform set of data points.

However, these 21 parameters were not independent from one another. To create a set of independent basis functions with which to describe the impedance responses, we described each impedance response of the Reference Set as a point in 21-dimensional space given by the 21 factors a_i. We used PCA to identify the orthogonal directions of maximum variance in the resulting 21-dimensional point cloud. As each of these direction vectors could be considered a set of 21 factors a_i, each of these direction vectors, or eigenvectors, corresponded to one impedance response component shape. We found that the first seven eigenvectors covered >97.5% of the total variance in the data (**Figure 4d**). We thus obtained a set of seven independent impedance response component shapes (eigenshapes), which we could multiply and sum to accurately reproduce all impedance responses. In effect, these seven eigenshape multiplication factors could be used as the seven parameters that uniquely characterize each impedance response.

Creating a visual description of pathway contributions to impedance signatures: "star plots"

For every ligand, impedance responses were obtained under control (vehicle pre-treated) conditions and in the presence of six different pathway inhibitors. Even with only a set of seven parameters to characterize each individual impedance response, this would have necessitated 49 parameters to describe the contribution of six pathways to a given ligand impedance signature. Therefore, we sought to develop a method that could describe the

qualitative changes induced by each of the six pathway inhibitors as a single measure, to thus produce six descriptors representing the complete impedance profiles for each ligand.

As a measure of the qualitative changes in a ligand's impedance response induced by a given pathway inhibitor, we chose the cosine distance between the vectors by the seven eigenshape factors describing the impedance responses generated in the control condition and each pathway inhibitor pre-treated condition, respectively:

$$d_{ctrl-inhib} = 1 - \frac{\sum_i a_{ctrl,i} a_{inhib,i}}{\sqrt{\sum_i a_{ctrl,i}^2 \sum_i a_{inhib,i}^2}}$$

where *d* is the cosine distance, and *a* are the seven parameters characterizing each impedance response.

The cosine distance has three attractive properties: (1) it is a bounded distance, taking on values between 0 and 2; (2) if two sets of parameters differ only in a linear manner (i.e. two impedance signatures have the same shape, but differ only in the magnitude of the response), the distance remains 0; and (3) when only a single parameter is very different, the distance becomes large, thus producing larger values for qualitative rather than quantitative changes in the impedance response upon inhibitor pre-treatment. Particularly this last property is not shared with other distance functions, such as Euclidean distance, which could not distinguish between the two cases.

Instead of 49 parameters to characterize the effect of pathway inhibitors on a ligand impedance signature, we were thus left with a set of 6 descriptors between 0 and 2. Plotting the six values as the six radii of a hexagon allowed us to visualize the impedance profiles as a "star plot". For ease of both visibility and calculation, we mapped the distance via a simple linear transformation so that a distance of 0 would correspond to a radius of 0.2 and a distance of 2 to a radius of 1. This produced a straightforward means to visually assess the impedance profiles of ligands.

Clustering impedance profiles

Visual inspection of the star plots suggested distinct functional groups of ligands. We hypothesized that a distance function with very similar qualities to the cosine distance, but that would avoid grouping very small stars (i.e. impedance signatures that were nearly unaffected by the addition of inhibitors), with large stars (i.e. impedance signatures that were noticeably affected by the addition of one or more pathway inhibitors). Thus, we proposed a modified cosine distance for comparing impedance profiles,

$$d_{p_j p_k} = 1 - \frac{\sum_i (ci_i^{(j)} + \delta)(ci_i^{(k)} + \delta)}{\sqrt{\sum_i (ci_i^{(j)} + \delta)^2 \sum_i (ci_i^{(k)} + \delta)^2}}$$

where $d_{p_j p_k}$ is the distance between ligand impedance profiles (i.e. difference between star plots), *j* and *k*, $ci_i^{(j)}$ is the cosine distance between control and the i^{th} inhibitor-treated impedance response of condition *j,* and δ is an offset that ensures a difference between small and large stars, but that doesn't significantly affect the distance between large stars. We find that δ=0.1 leads to a very good grouping of ligands of known functional classes (**Figure 4f**). With this distance measure, we performed hierarchical complete linkage clustering, which tends to create compact clusters.

REFERENCES

Anson, B. D., Kolaja, K. L., & Kamp, T. J. (2011). Opportunities for use of human iPS cells in predictive toxicology. *Clinical pharmacology and therapeutics*, *89*(5), 754–8. doi:10.1038/clpt.2011.9

Chidiac, P., Hebert, T. E., Valiquette, M., Dennis, M., & Bouvier, M. (1994). Inverse agonist activity of beta-adrenergic antagonists. *Molecular pharmacology*, *45*(3), 490–9.

Galandrin, S., & Bouvier, M. (2006). Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol*, *70*(5), 1575–1584. doi:mol.106.026716 [pii] 10.1124/mol.106.026716

Guimaraes, S., & Moura, D. (2001). Vascular Adrenoceptors: An Update. *Pharmacol. Rev.*, *53*(2), 319–356.

Johnson, R., Webb, J. G., Newman, W. H., & Wang, Z. (2006). Regulation of human vascular smooth muscle cell migration by beta-adrenergic receptors. *The American surgeon*, *72*(1), 51–4.

Kaya, A. I., Onaran, H. O., Ozcan, G., Ambrosio, C., Costa, T., Balh, S., & Ugur, O. (2012). Cell Contact-Dependent Functional Selectivity of β2-Adrenergic Receptor Ligands in Stimulating cAMP Accumulation and Extracellular Signal-Regulated Kinase (ERK) Phosphorylation. *The Journal of biological chemistry*. doi:10.1074/jbc.M111.301820

Kenakin, T., & Christopoulos, A. (2012). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nature reviews. Drug discovery*, *advance on*. doi:10.1038/nrd3954

Liu, J. J., Horst, R., Katritch, V., Stevens, R. C., & Wüthrich, K. (2012). Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. *Science (New York, N.Y.)*, *335*(6072), 1106–10. doi:10.1126/science.1215802

Patel, C. B., Noor, N., & Rockman, H. A. (2010). Functional selectivity in adrenergic and angiotensin signaling systems. *Molecular pharmacology*, *78*(6), 983–92. doi:10.1124/mol.110.067066

Peters, M. F., & Scott, C. W. (2009). Evaluating Cellular Impedance Assays for Detection of GPCR Pleiotropic Signaling and Functional Selectivity. *Journal of Biomolecular Screening*, *14*(3), 246–255. doi:10.1177/1087057108330115

Pincus, Z., & Theriot, J. A. (2007). Comparison of quantitative methods for cell-shape analysis. *Journal of microscopy*, *227*(Pt 2), 140–56. doi:10.1111/j.1365-2818.2007.01799.x

Rajagopal, S., Ahn, S., Rominger, D. H., Gowen-MacDonald, W., Lam, C. M., Dewire, S. M., … Lefkowitz, R. J. (2011). Quantifying ligand bias at seven-transmembrane receptors. *Molecular pharmacology*, *80*(3), 367–77. doi:10.1124/mol.111.072801

Reiner, S., Ambrosio, M., Hoffmann, C., & Lohse, M. J. (2010). Differential signaling of the endogenous agonists at the {beta}2-adrenergic receptor. *The Journal of biological chemistry*, *285*(46), 36188 –36198. doi:10.1074/jbc.M110.175604

Schröder, R., Janssen, N., Schmidt, J., Kebig, A., Merten, N., Hennen, S., … Kostenis, E. (2010). Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. *Nature biotechnology*, *28*(9), 943–949. doi:10.1038/nbt.1671

Stallaert, W., Christopoulos, A., & Bouvier, M. (2011). Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors. *Expert Opinion on Drug Discovery*, *6*(8), 811–825. doi:10.1517/17460441.2011.586691

Stallaert, W., Dorn, J. F., van der Westhuizen, E., Audet, M., & Bouvier, M. (2012). Impedance Responses Reveal β(2)-Adrenergic Receptor Signaling Pluridimensionality and Allow Classification of Ligands with Distinct Signaling Profiles. (C. V. Rao, Ed.)*PloS one*, *7*(1), e29420. doi:10.1371/journal.pone.0029420

Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., … Mailman, R. B. (2007). Functional Selectivity and Classical Concepts of Quantitative Pharmacology. *Pharmacology*, *320*(1), 1–13. doi:10.1124/jpet.106.104463.Receptor

Xi, B., Wang, T., Li, N., Ouyang, W., Zhang, W., Wu, J., … Abassi, Y. A. (2011). Functional cardiotoxicity profiling and screening using the xCELLigence RTCA Cardio System. *Journal of laboratory automation*, *16*(6), 415–21. doi:10.1016/j.jala.2011.09.002

Yu, N., Atienza, J. M., Bernard, J., Blanc, S., Zhu, J., Wang, X., … Abassi, Y. a. (2006). Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Analytical chemistry*, *78*(1), 35–43. doi:10.1021/ac051695v

FIGURES

FIGURE 1

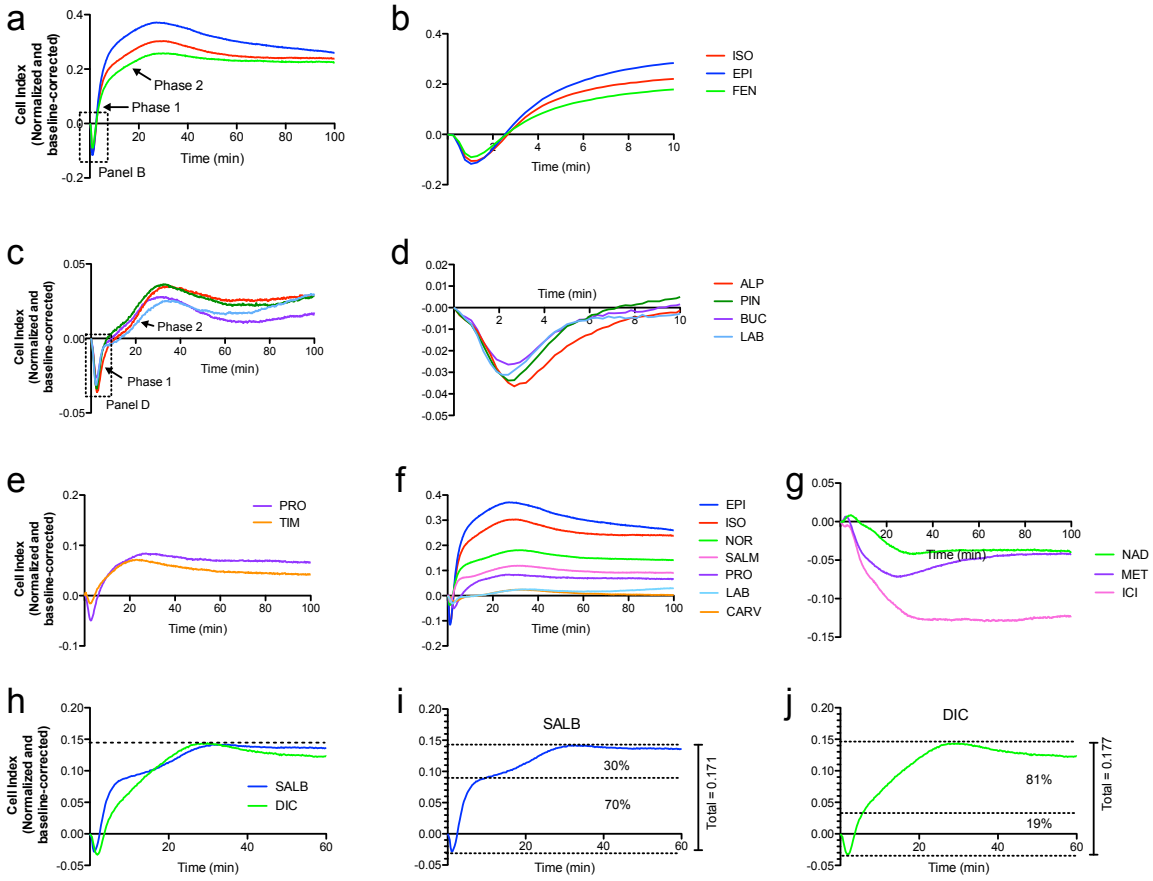


Figure 1: Impedance signatures of Reference Set compounds. Impedance signatures consist of several, distinct features that can differ independently among Reference Set compounds (1 µM each). **(a,b)** Some ligands elicit a transient negative phase and two distinct ascending phases before reaching a maximum response, while **(c,d)** others elicit a more sustained negative phase and two distinct ascending phases. **(e)** Some ligands elicit only a single ascending phase. **(f)** Ligands can differ in their maximum response. **(g)** Other ligands elicit a predominantly negative response. **(h)** Ligands can also differ in the proportions of the ascending phases despite reaching a similar maximum response. Data represent the mean of at least three independent experiments. Impedance signatures for the complete Reference Set are presented in Supplementary Figure 1. Abbreviations: Isoproterenol (ISO), epinephrine (EPI), fenoterol (FEN), alprenolol (ALP), pindolol (PIN), bucindolol (BUC) and labetalol (LAB), propranolol (PRO), timolol (TIM), norepinephrine (NOR), salmeterol (SALM), carvedilol (CARV), dicholorisoproterenol (DIC).

FIGURE 2

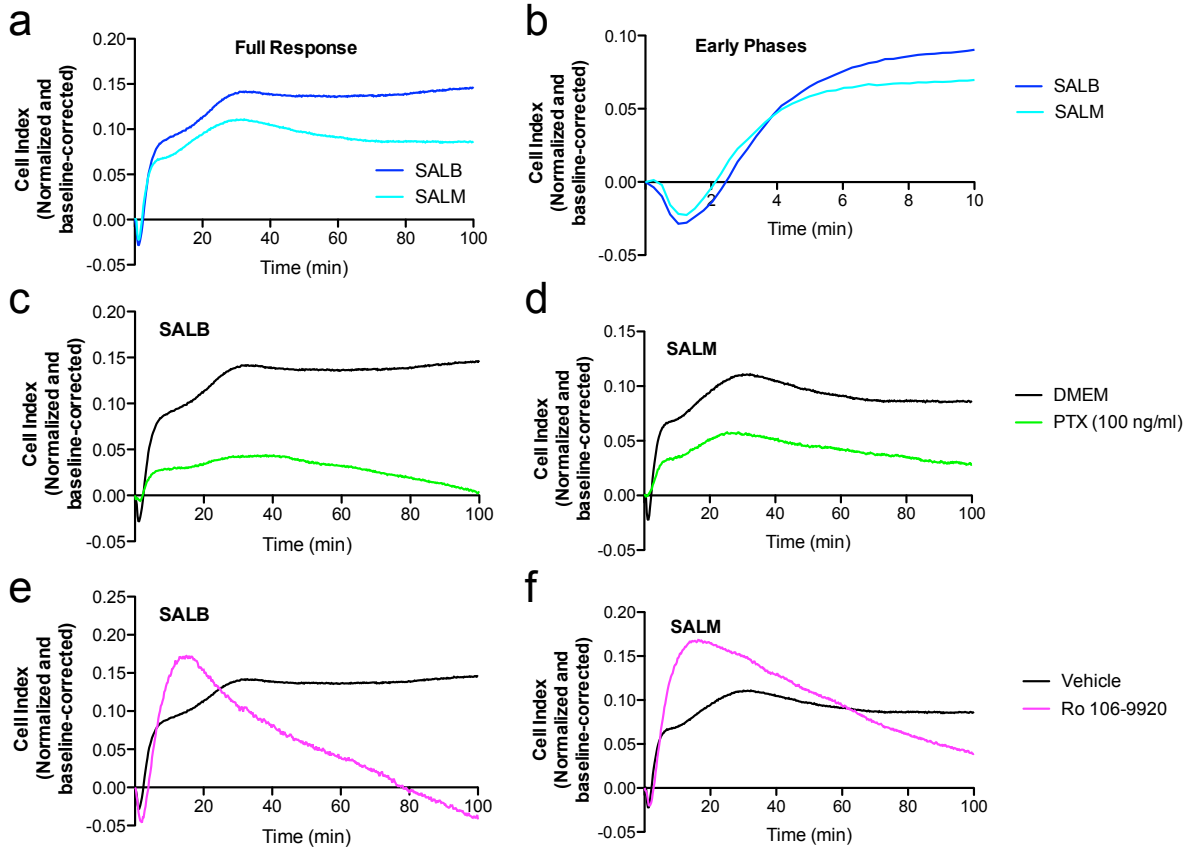


Figure 2: Use of pathway-selective inhibitors reveals additional distinctions in SALB and SALM impedance responses. (a,b) SALB and SALM (1 μM each) elicit very similar impedance signatures consisting of a transient negative phase, two distinct ascending phases and a similar maximum response. **(c,d)** Pre-treatment with the Gi inhibitor pertussis toxin (PTX, 100 ng/ml, 18 h) inhibits a greater proportion of the SALB response compared to SALM. **(e,f)** Pre-treatment with the NF-κB pathway inhibitor Ro 106-9920 (12.5 μM, 30 min) leads to a much greater increase in the first ascending phase and a more sustained maximum response for SALM and a complete loss of the second ascending phase for both ligands. Data represent the mean of at least three independent experiments. Abbreviations: salbutamol (SALB), salmeterol (SALM).

FIGURE 3

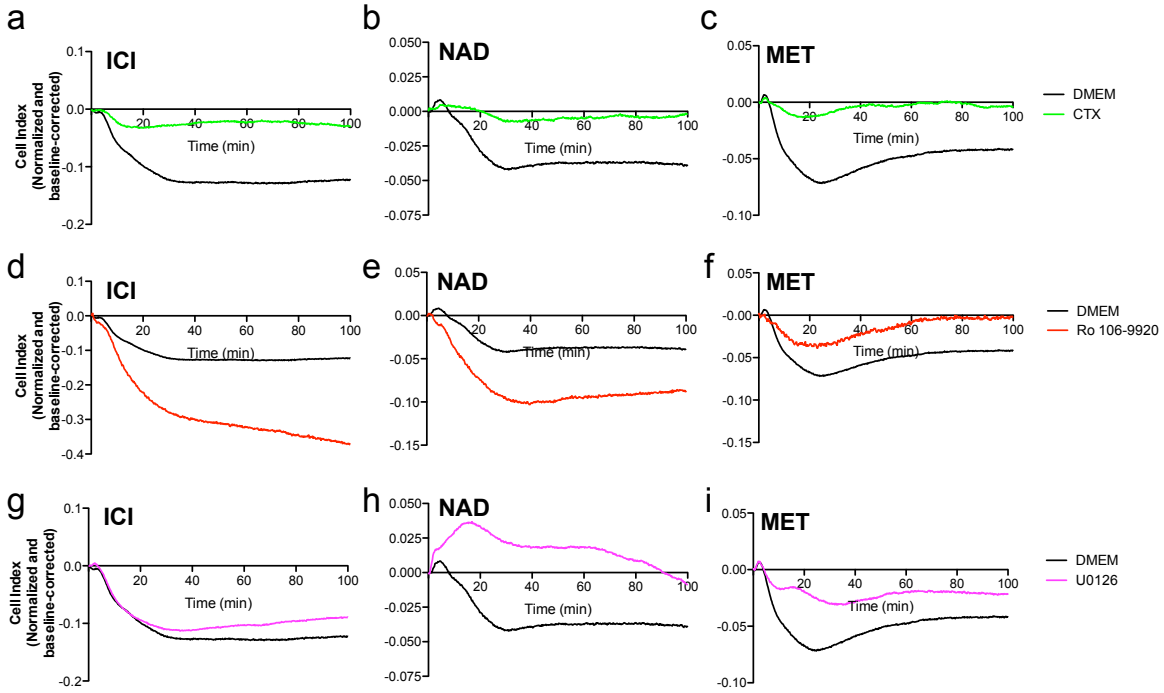


Figure 3: ICI, NAD and MET differ in the contribution of Gs, NF-κB and ERK1/2 signalling to their impedance signatures. (a,b,c) ICI, NAD and MET (1 µM each) elicit similar, predominantly negative impedance responses that are completely inhibited following pre-treatment with the Gs inhibitor cholera toxin (CTX, 200 ng/ml, 18 h). **(d,e,f)** Pre-treatment with the NF-κB inhibitor Ro 106-9920 (12.5 µM, 30 min) leads to more negative impedance responses for ICI and NAD, the MET impedance response becomes more positive. **(g,h,i)** Pre-treatment with the ERK1/2 pathway inhibitor U0126 (5 µM, 30 min) has no effect on the ICI impedance response yet leads to more positive responses for NAD and MET, increasing the NAD response to predominantly positive. Data represent the mean of at least three independent experiments. Abbreviations: ICI 118,551 (ICI), nadolol (NAD), metoprolol (MET).

FIGURE 4

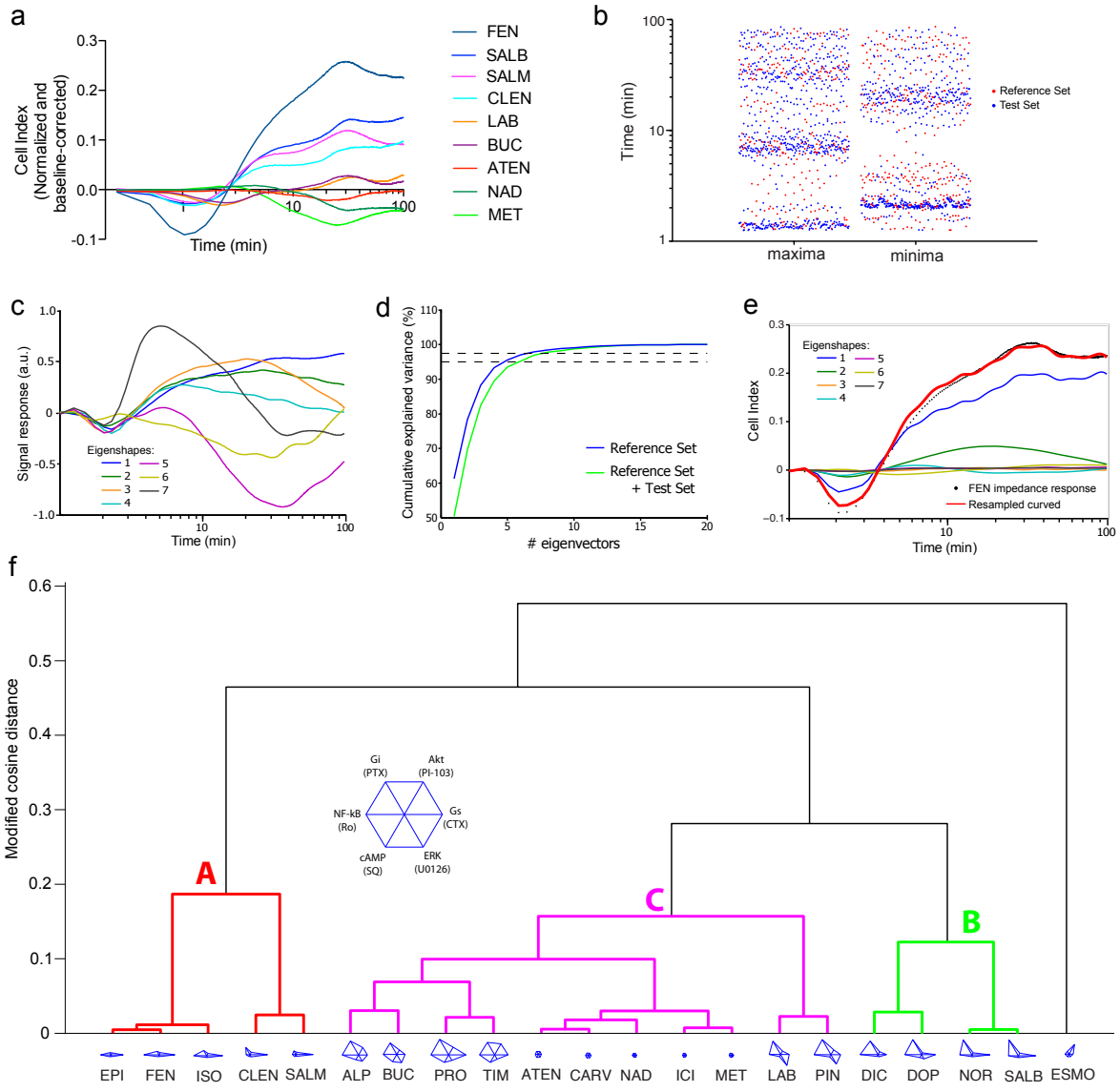
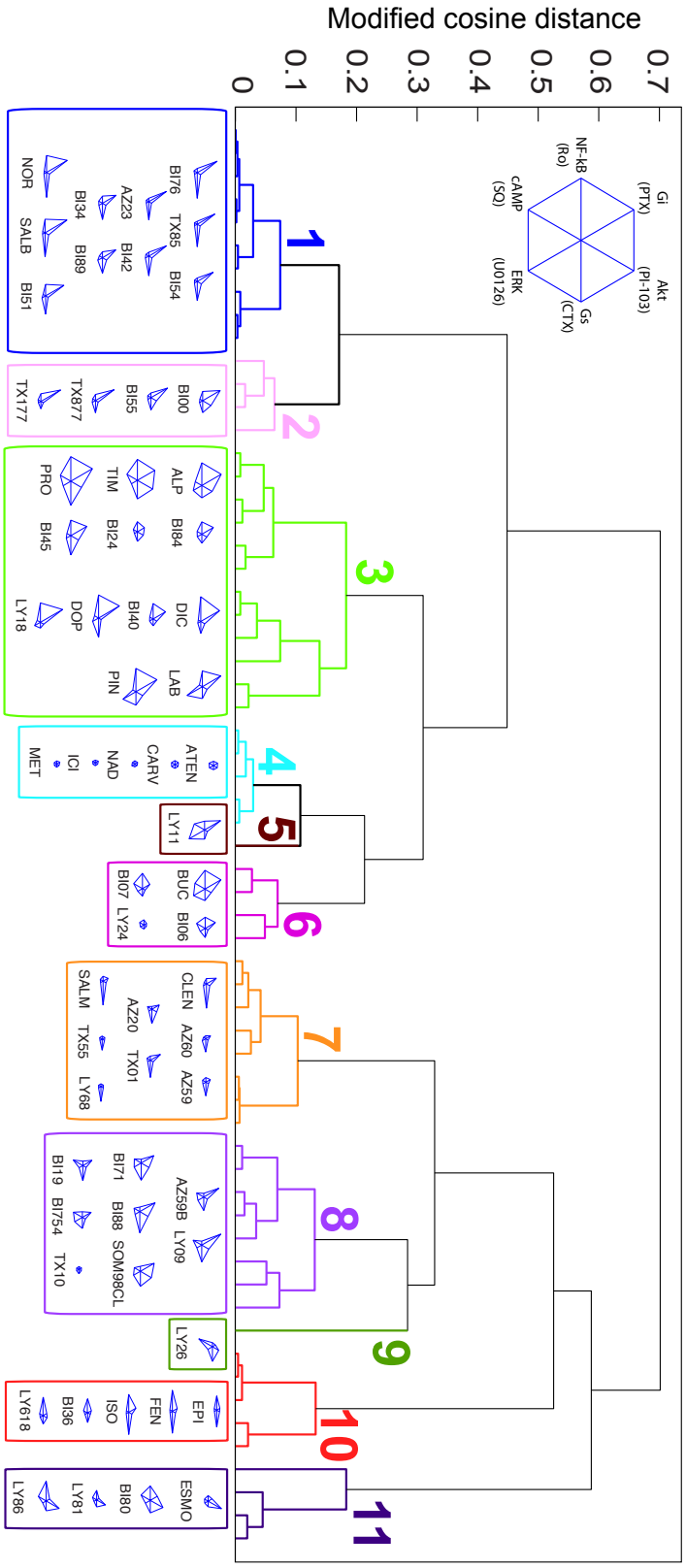

a
Cell Index (Normalized and baseline-corrected)
Time (min)
FEN
SALB
SALM
CLEN
LAB
BUC
ATEN
NAD
MET
b
Time (min)
Reference Set
Test Set
maxima
minima
c
Signal response (a.u.)
Eigenshapes:
Time (min)
d
Cumulative explained variance (%)
Reference Set
Reference Set + Test Set
eigenvectors
e
Cell Index
Eigenshapes:
FEN impedance response
Resampled curved
Time (min)
f
Modified cosine distance
Gi (PTX)
Akt (PI-103)
NF-kB (Ro)
Gs (CTX)
cAMP (SQ)
ERK (U0126)
A
B
C
EPI FEN ISO CLEN SALM ALP BUC PRO TIM ATEN CARV NAD ICI MET LAB PIN DIC DOP NOR SALB ESMO

Figure 4: Computational analysis of the Reference Set impedance profiles allows clustering of compounds based on their impedance profiles. (a) Log-time transformation of the impedance signatures reveals wave-like responses. **(b)** Log-time transformed impedance responses exhibit local oscillations at similar time regimes (Reference Set: blue dots; Test Set: red dots). **(c,d)** Seven distinct eigenshapes were obtained (c) and used to resample the log-time transformed impedance responses, providing coverage of >97.5% of the total variance for all impedance responses (d). **(e)** Log-time transformed impedance responses can be resampled using linear combinations of the seven eigenvectors obtained, as shown here for fenoterol (FEN) (black dots: measured FEN impedance response; Red line: resampled FEN response). **(f)** Star plots were obtained for each of the Reference Set compounds (1 μM each). The distance between the center of the star and each corner represents the sensitivity of the impedance signature to each of the pathway inhibitors (see *inset* for Legend). Compounds were then clustered according to the similarity of their star plots. In panel **a**, data represent the mean of at least three independent experiments and, for clarity, only a subset of Reference set compounds are shown. Please refer to *Methods* for a complete description of the analysis used.

FIGURE 5
a


Modified cosine distance
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
NF-kB (Ro)
Gi (PTX)
cAMP (SQ)
Akt (PI-103)
ERK (U0126)
Gs (CTX)
1
2
3
4
5
6
7
8
9
10
11
BI76 TX85 BI54 AZ23 BI42 BI34 BI89 NOR SALB BI51
BI00 BI55 TX877 TX177
ALP BI84 TIM BI24 PRO BI45 DIC BI40 DOP LY18 LAB PIN
ATEN CARV NAD ICI MET
LY11
BUC BI06 BI07 LY24
CLEN AZ60 AZ59 AZ20 TX01 SALM TX55 LY68
AZ59B LY09 BI71 BI88 SOM98CL BI19 BI754 TX10
LY26
EPI FEN ISO BI36 LY618
ESMO BI80 LY81 LY86

b

GROUP	Compound	CTX Gs	PTX Gi	Ro NF-kB	PI-103 Akt	SQ22536 cAMP	U0126 ERK1/2	Pre-treatment Pathway
1	BI76	1.93	1.71	0.14	0.11	0.09	0.03	
	TX85	1.68	1.48	0.03	0.06	0.04	0.03	
	BI54	1.38	1.32	0.08	0.18	0.03	0.04	
	AZ23	1.32	1.49	0.06	0.04	0.02	0.02	
	BI42	1.45	1.50	0.09	0.09	0.07	0.10	
	BI34	1.15	1.11	0.38	0.12	0.14	0.13	
	BI89	1.08	1.08	0.28	0.18	0.07	0.22	
	NOR	1.88	1.80	1.20	0.06	0.01	0.03	
	SALB	1.81	1.92	0.92	0.13	0.01	0.01	
	BI51	1.31	1.55	0.75	0.02	0.03	0.12	
2	BI00	0.78	1.49	0.55	0.59	0.05	0.09	
	BI55	0.82	1.39	0.18	0.32	0.10	0.09	
	TX877	0.81	1.59	0.30	0.03	0.05	0.10	
	TX177	0.41	1.69	0.42	0.01	0.06	0.03	
3	ALP	0.81	1.67	1.68	0.78	0.41	0.61	
	BI84	0.54	0.90	0.82	0.27	0.23	0.22	
	TIM	1.34	1.46	1.54	1.26	0.80	0.36	
	BI24	0.58	0.47	0.63	0.35	0.18	0.06	
	PRO	1.80	1.88	1.85	0.82	0.79	0.52	
	BI45	1.41	1.34	1.08	0.53	0.16	0.21	
	DIC	0.77	1.61	1.87	0.17	0.02	0.05	
	BI40	0.35	0.98	1.08	0.14	0.10	0.12	
	DOP	1.41	1.81	1.72	0.04	0.17	0.34	
	LY18	0.27	1.94	1.42	0.10	0.10	0.34	
	LAB	0.79	1.58	1.10	0.25	0.06	1.26	
	PIN	0.90	1.82	1.74	0.44	0.08	1.03	
4	ATEN	0.12	0.19	0.14	0.15	0.20	0.16	
	CARV	0.10	0.11	0.12	0.09	0.11	0.10	
	NAD	0.02	0.05	0.06	0.01	0.02	0.07	
	ICI	0.02	0.07	0.01	0.01	0.03	0.00	
	MET	0.05	0.09	0.04	0.01	0.02	0.01	
5	LY11	0.56	1.33	0.52	0.05	0.78	1.29	
6	BUC	0.75	1.33	1.35	0.39	0.75	0.89	
	BI06	0.55	0.77	0.72	0.05	0.62	0.27	
	BI07	0.40	0.49	1.08	0.04	0.63	0.24	
	LY24	0.12	0.03	0.21	0.01	0.18	0.11	
7	CLEN	1.82	0.73	0.23	0.09	0.01	0.08	
	AZ60	0.90	0.24	0.02	0.00	0.01	0.12	
	AZ59	0.93	0.21	0.16	0.03	0.11	0.08	
	AZ20	0.99	0.48	0.02	0.24	0.22	0.08	
	TX01	1.23	0.74	0.13	0.04	0.10	0.04	
	SALM	1.79	0.26	0.19	0.06	0.06	0.02	
	TX55	0.71	0.03	0.03	0.01	0.03	0.00	
	LY68	1.03	0.01	0.02	0.00	0.01	0.01	
8	AZ59B	0.91	1.24	0.08	0.13	0.47	0.34	
	LY09	1.19	1.63	0.12	0.46	0.61	0.25	
	BI71	1.23	1.00	0.10	0.55	0.45	0.46	
	BI88	1.56	1.28	0.16	0.59	0.34	0.24	
	SOM98CL	1.05	1.12	0.20	0.85	0.38	0.45	
	BI19	1.07	0.66	0.15	0.09	0.71	0.15	
	BI754	0.71	0.63	0.25	0.06	0.64	0.52	
	TX10	0.15	0.02	0.01	0.01	0.01	0.00	
9	LY26	1.02	0.07	0.12	0.39	1.09	0.23	
10	EPI	1.22	0.08	0.97	0.05	0.02	0.01	
	FEN	1.63	0.24	1.54	0.07	0.02	0.02	
	ISO	1.65	0.47	1.29	0.08	0.01	0.03	
	BI36	0.60	0.13	0.96	0.03	0.18	0.05	
	LY618	0.49	0.11	1.31	0.15	0.13	0.08	
11	ESMO	0.25	0.15	0.24	1.07	0.14	0.12	
	BI80	0.83	0.46	0.99	1.00	0.68	0.25	
	LY81	0.38	0.01	0.58	0.63	0.18	0.04	
	LY86	0.60	0.11	1.45	1.03	0.55	0.12	

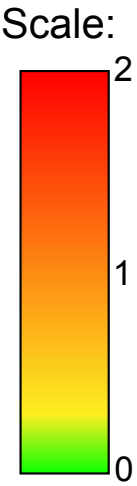


Figure 5: Impedance profiling predicts functional differences among an uncharacterized compound library. (a) Star plots were obtained for Test Set compounds (1 μM each) and, with the Reference Set, all compounds were clustered as described in *Methods* (see *inset* for Legend). **(b)** The magnitude of the descriptors producing the star plots in panel **a**. The clustering obtained reflects differences in pathway contributions to each impedance response.

FIGURE 6

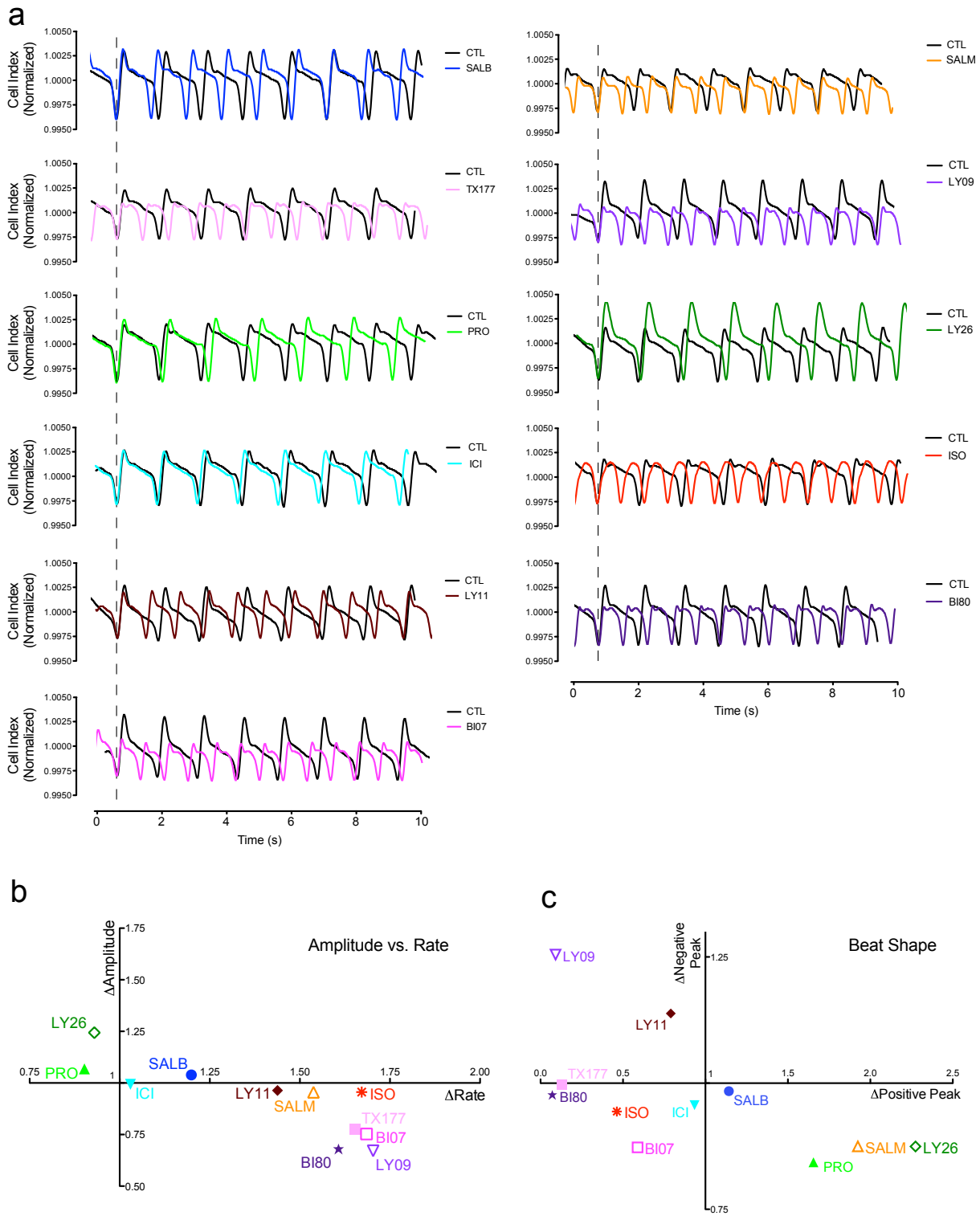
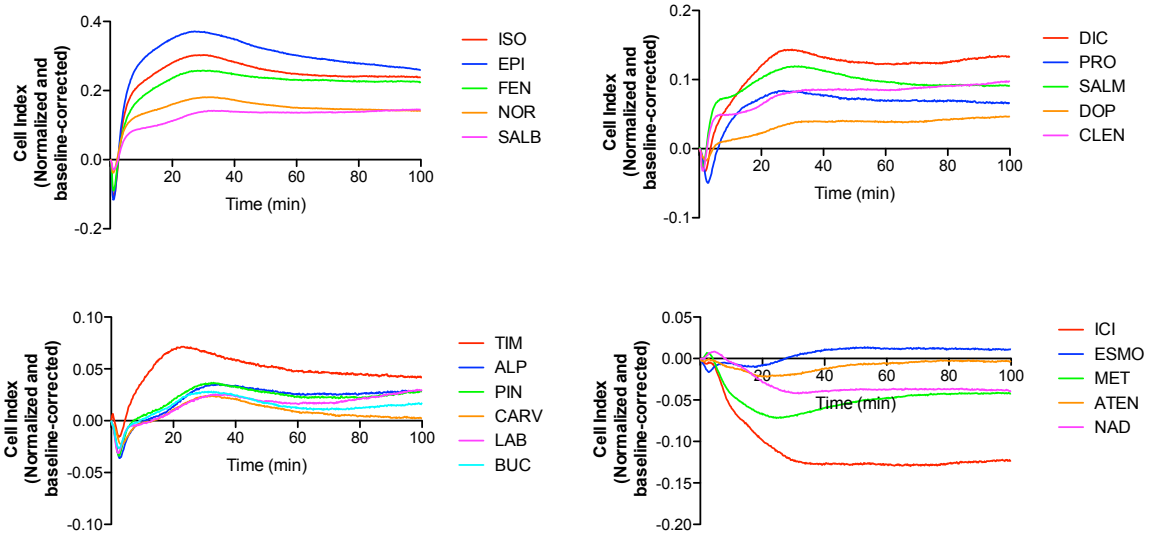

a
Cell Index (Normalized)
CTL
SALB
TX177
PRO
ICI
LY11
BI07
SALM
LY09
LY26
ISO
BI80
Time (s)
b
Amplitude vs. Rate
ΔAmplitude
ΔRate
c
Beat Shape
ΔNegative Peak
ΔPositive Peak

Figure 6: Different compound classes elicit distinct effects on cardiomyocyte contractility. (a) Representative ligands from each of the compound classes identified by impedance profiling were assessed for their effects on cardiomyocyte contractility. Traces represent the effect of compounds (1 μM each, coloured trace) 1 h post-stimulation compared to pre-stimulation control traces (black trace). **(b)** The effect of compounds on normalized beat rate and amplitude. **(c)** The effect of compounds on beat shape as described by changes in the positive peak and the negative peak of the contractile response. Abbreviations: salbutamol (SALB), propranolol (PRO), ICI 188,551 (ICI), salmeterol (SALM), isoproterenol (ISO).

Supplementary Table 1: Pharmacological activity of Reference Set compounds.
Adapted from Stallaert et al. (2012); Reiner et al. (2010); Chidiac et al. (1994); and Kaya et al. (2012).

Compound	Abbreviation	cAMP	ERK1/2	Reference
Epinephrine	EPI	Full agonist	Full agonist	Stallaert et al. (2012)
Isoproterenol	ISO	Full agonist	Full agonist	Stallaert et al. (2012)
Fenoterol	FEN	Strong partial agonist	*nd*	Reiner et al. (2010)
Norepinephrine	NOR	Weak partial agonist	Full agonist	Kaya et al. (2012)
Salbutamol	SALB	Full agonist	Strong partial agonist	Stallaert et al. (2012)
Dichloroisoproterenol	DIC	Inverse agonist	*nd*	Chidiac et al. (1994)
Clenbutarol	CLEN	Strong partial agonist	Strong partial agonist	Kaya et al. (2012)
Salmeterol	SALM	Full agonist	Strong partial agonist	Stallaert et al. (2012)
Alprenolol	ALP	Weak partial agonist	Weak partial agonist	Stallaert et al. (2012)
Pindolol	PIN	Weak partial agonist	Weak partial agonist	Stallaert et al. (2012)
Dopamine	DOP	Weak partial agonist	Strong partial agonist	Kaya et al. (2012)
Bucindolol	BUC	Weak partial agonist	Weak partial agonist	Stallaert et al. (2012)
Labetalol	LAB	Weak partial agonist	Weak partial agonist	Stallaert et al. (2012)
Atenolol	ATEN	Inverse agonist	Inverse agonist	Stallaert et al. (2012)
Carvedilol	CARV	Inverse agonist	Weak partial agonist	Stallaert et al. (2012)
Esmolol	ESMO	*nd*	*nd*	
ICI 118,551	ICI	Inverse agonist	Inverse agonist	Stallaert et al. (2012)
Metoprolol	MET	Inverse agonist	Inverse agonist	Stallaert et al. (2012)
Nadolol	NAD	Inverse agonist	Inverse agonist	Stallaert et al. (2012)
Propranolol	PRO	Inverse agonist	Weak partial agonist	Stallaert et al. (2012)
Timolol	TIM	Inverse agonist	Inverse agonist	Stallaert et al. (2012)

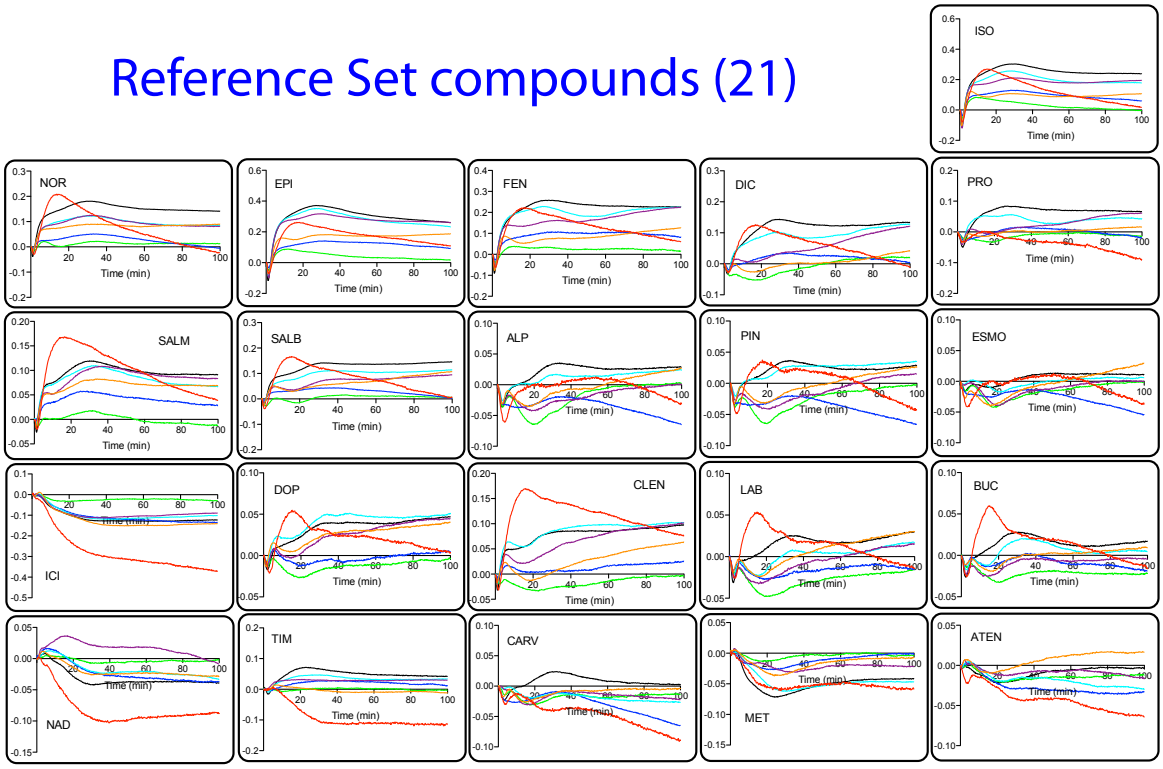
Supplementary Figure 1



Supplementary Figure 1: Impedance signatures of the Reference Set compounds. All compounds were assayed at 1 µM each. Data represent the mean of at least three independent experiments.

Supplementary Figure 2

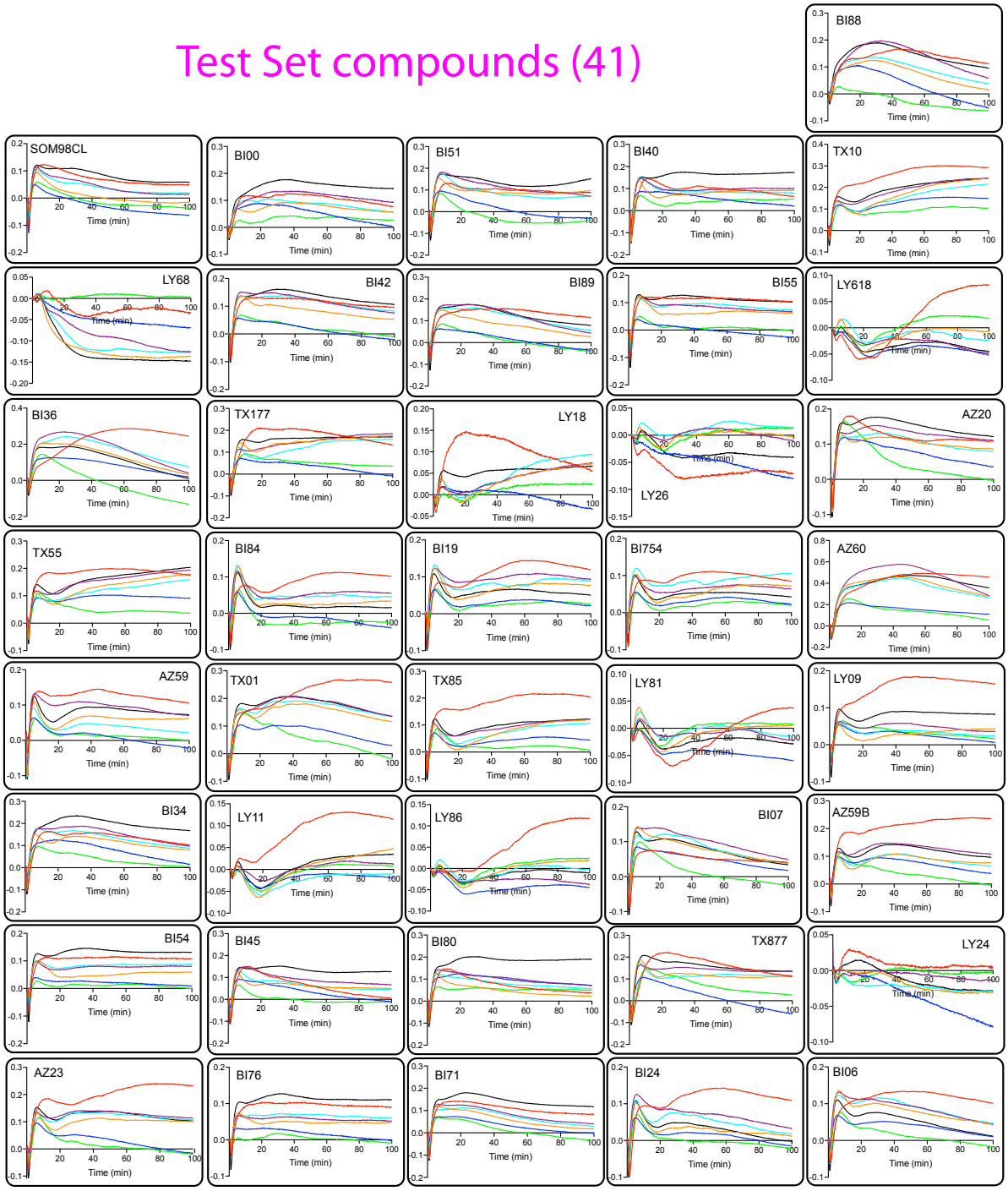
a



6 pathway-selective inhibitors

- No Pre-treatment
- CTX (200 ng/ml)
- PTX (100 ng/ml)
- SQ22536 (100 μM)
- U0126 (5 μM)
- PI-103 (100 nM)
- Ro 106-9920 (12.5 μM)

b



Supplementary Figure 2: Impedance profiles of complete Reference Set and Test Set. **(a)** References Set and **(b)** Test Set compounds (1 µM each) were pre-treated with cholera toxin (CTX, 200 ng/ml, 18h), pertussis toxin (PTX, 100 ng/ml, 18h), SQ22536 (100 µM, 30 min), U0126 (5 µM, 30 min), PI-103 (100 nM, 30 min), Ro 106-9920 (12.5 µM, 30 min) or vehicle (DMEM). Data represent the mean of at least three independent experiments.

DISCUSSION

1. A return from reductionism

With the incredible technological advancements of the previous few decades, especially those made in areas such as molecular biology, pharmacological research has evolved to take advantage of the reductionist approaches made possible. The creation of immortalized cell lines, for instance, availed an abundant source of cells in which the activity of many drugs could be compared across a genetically identical background. Developments in protein purification and cloning led to the identification and characterization of many GPCRs (and subtypes) as well as the effectors that couple these receptors to a cellular response. Altogether, the heterologous expression of a given GPCR subtype in a specific immortalized cell line provided researchers a means to study the pharmacology of a receptor and its ligands with exceptional specificity and resolution. While these techniques have provided an extraordinary wealth of information the signalling networks of GPCRs, the discoveries made in these non-native systems are not always conserved in cells endogenously expressing the receptor and its effectors at physiological levels. Indeed, the stoichiometry of receptors and effectors is an important aspect of the functionality of signalling networks and their influence on the cellular response that is elicited. In fact, these differences determine the cell-type specificity of the responses generated by receptor stimulation and could explain the many instances of contradiction in the literature regarding the mechanisms underlying a given cellular response. Thus, while the utility of reductionist approaches make them indispensible in the study of GPCR signalling, the applicability of the results obtained must be subsequently validated in a model that more closely resembles the native signalling context of the receptor.

The recent development of label-free techniques such as cellular impedance and DMR, on the other hand, recalls the classical approach of using isolated tissues to study the holistic impact of drugs targeting GPCRs and is an alternative approach to the reductionist techniques commonly used today. While the pluridimensionality of GPCR signalling had yet to be recognized (or even imagined) at the time, the use of isolated tissues to study drug responses unknowingly incorporated the impact of functional selectivity on physiological

responses in, for example, the ileum (Stephenson, 1956), uterus (Gaddum et al. 1955), stomach (Black, Fisher, and Smith 1958) or heart (Levy & Richards, 1965), to name a few. While these classical techniques provide an integrative assessment of ligand activity towards a functional response, they are not compatible with many of the modern techniques (e.g. genetic manipulation, high throughput screening, etc.) used to study the molecular determinants of GPCR signalling or discover drugs targeting these receptors. Both cellular impedance and DMR possess the integrative properties of these classical approaches while also being amenable to modern molecular pharmacology/drug discovery techniques. Receptor specificity can be determined, for instance, by assessing the responses generated in cell lines heterologously expressing the receptor of interest compared to the parental cell line. In addition, with the use of pharmacological inhibitors, the signalling components underlying a given response can be determined with relative ease. Given the ability of these approaches to be performed in microtitre plates, they also provide a fairly rapid assessment of signalling activity for a large number of compounds and/or conditions.

While the integrative nature of these techniques does offer several advantages in the study of GPCR signalling, it also increases the potential for off-target effects to influence the responses obtained. Indeed, this is a problem common to most approaches to study signalling, but because label-free techniques measure the overall cellular response to receptor activation they are more likely to be influenced by any off-target effects of a given inhibitor. Indeed, it is difficult to distinguish the contribution of the effector of interest from any other cellular components affected by the pathway inhibitor. In Articles 1 and 3 presented in this thesis (pages 63 and 140, respectively), concentrations of pathway inhibitors were used that provided as much selectivity as could be predicted based on any reported off-target effects; however, we cannot completely rule out the contribution of off-target effects in the results obtained. Although, in the context of ligand screening, any potential influence of off-target effects will be subjected across the entire library. Thus if the goal is to functionally differentiate ligands, differences in the effect of inhibitor treatment, at the effector of interest or not, will still act to distinguish ligands with different mechanisms of action.

Furthermore, while label-free approaches are technically amenable to genetic manipulation to study the role of effectors with great specificity, there exist some confounding factors that limit the use and interpretability of genetic knockdown or overexpression in this context. The primary concern is that since ligand treatment would normally occur 48-72 hours following transfection, the cell has ample time to adapt to the manipulation and thus the overall state of the cell could be fundamentally changed in a way that is not specific to the modulation in expression/activity of the effector of interest. For example, knockdown of a given effector might lead to the upregulation of other signalling proteins/pathways as an adaptive mechanism to ensure a given cellular response to receptor activation. This is less of an issue with assays that measure a single signalling outcome (e.g. activation of a given kinase), as any potential influence of adaptive mechanisms on the results obtained would be limited only to those that alter the signalling network upstream of the response measured. In integrative assays such as cellular impedance, any changes in the overall signalling network of the receptor could fundamentally change the measured response to receptor stimulation, including effects that are directly attributable to the effector of interest and those that are part of a greater adaptive response. For this reason, in Articles 1 and 3 we elected to use acute pharmacological inhibition of effectors (i.e. 30 minute pre-treatment), as an attempt to reduce the influence of any adaptive responses on the results obtained. Therefore, we are limited to studying the role of signalling components for which an effective inhibitor is available. For this reason, we could not study the impact of β-arrestin signalling directly since an inhibitor has not yet been identified for this important GPCR effector. In Article 3, the influence of β-arrestin signalling is instead assessed indirectly using an inhibitor of NF-κB signalling, which is known to be modulated by the β_2AR in a β-arrestin-dependent manner (Gao et al., 2004). The development of an effective β-arrestin inhibitor (see Annex I, page *xvii*) should permit an exploration of the role of this multifunctional protein in the overall cellular response to GPCR activation.

Another potential limitation of the use of cellular impedance to study GPCR signalling networks is the measurement of impedance itself. The readout is simply a measurement of changes in the adhesion of cells to the microelectrode sensors that line the bottom of the

microtitre plates used. Therefore, any signalling events that converge on changes in cell morphology or adhesion will theoretically contribute to the responses obtained. However, we cannot rule out that certain signalling events might be overlooked because they do not influence the changes in morphology or adhesion measured by this assay. However, in Articles 1 and 3, impedance measurements were found to integrate the contribution of all of the known β_2AR effectors assessed. In fact, because of the ability of impedance to detect the involvement of any pathway that influences these cellular responses, a novel Ca^{2+} mobilization pathway was identified for the β_2AR. In Article 2 (page 110), it is revealed that this Ca^{2+} response results from the transactivation of P2Y11 purinergic receptors that are co-expressed in the HEK293 cells used in the study. Using reductionist approaches, we identified that β_2AR-dependent activation of G_s leads to the release of ATP to the extracellular compartment, which in turn activates P2Y11 receptors to initiate a G_q- and IP_3-dependent release of Ca^{2+} from intracellular stores. These results demonstrate the complementarity of label-free and reductionist approaches, with the ability of label-free techniques to generate new hypotheses that can subsequently be assessed using more traditional assays.

Altogether, label-free techniques offer several significant advantages that complement the current use of reductionist approaches to study GPCR signalling. The combined use of the two provides a powerful means to both assess the influence of signalling events on the overall cellular response to receptor stimulation and validate the individual effectors involved with a high degree of precision, making label-free techniques a powerful addition to the current drug discovery repertoire.

2. The use of cellular impedance in drug discovery

As previously discussed (see INTRODUCTION, *Section 7.3*, page 57), the ability to simultaneously monitor multiple signalling pathways in a single assay format provides several advantages for drug discovery over assays that measure only a single signalling outcome. Articles 1 and 3 represent the first attempts to exploit the integrative nature of impedance measurements to screen for functionally selective compounds targeting GPCRs. However, label-free techniques will probably not replace single-endpoint-defined assays as

the first-pass screen in drug discovery campaigns. While the 96- and 384-well formats of impedance-based systems do allow for a relatively high throughput (certainly much higher than assessing ligand activity in isolated tissues), neither possesses the throughput that assays based on monitoring a single signalling outcome provide (e.g. Ca^{2+}-sensitive fluorescent dyes, BRET- or FRET-based biosensors, etc.). By measuring a single signalling event after a relatively short compound treatment (e.g. 5-15 minutes), these other assays can be used to rapidly assess huge compound libraries, providing a coarse assessment of pharmacological activity (e.g. agonist or antagonist) at a receptor for millions of compounds in a relatively short amount of time. Cellular impedance often requires measurements of at least an hour, thus greatly increasing the time necessary to screen a large compound library or restricting the screen to a far fewer number of compounds. In addition, single outcome screens produce a single value to represent the pharmacological activity of a compound, providing an easy and rapid means to identify compounds targeting the receptor of interest. As demonstrated in this thesis, extracting the pharmacological significance of an impedance response requires a fairly complex computational analysis, which, at this stage would be impractical for a large compound library. While the pervasiveness of ligand functional selectivity at GPCRs increases the risk of missing compounds that target the receptor but do not modulate the specific pathway assessed in the screen, single outcome screening necessarily places an emphasis on throughput at the cost of potential false-negatives.

The use of label-free approaches, such as the impedance profiling presented in this thesis, could instead be valuable as a secondary screen of ligand signalling activity, particularly when the primary screen involves only a single assessment of compound pharmacological activity. After a primary high-throughput screen, compounds identified to target the receptor of interest ("*hits*") could subsequently be screened in a label-free system to obtain a more thorough representation of ligand signalling activity. Using impedance profiling, for example, the signalling profiles of hits could be assessed, visualized and clustered to reveal distinct functional classes of ligands among the hits obtained. Multiple hits representing different ligand classes could be validated and then further assessed in pre-clinical (and

perhaps clinical) studies to identify the compound with the best overall pharmacological profile for treatment of the disease in question.

Two different computational approaches were developed in the course of this thesis to cluster compounds based on their impedance responses. In Article 1, differences in ligand impedance signatures were quantified by measuring the area contained between two impedance responses using a method based on Euclidean distance. This approach allowed compounds to be clustered into groups based on their overall impedance responses, which were found to strongly correlate with the signalling profiles of the ligands towards the canonical cAMP and ERK1/2 pathways of the β_2AR. In Article 3, however, we sought to differentiate compounds not only by their overall impedance signatures but also by differences in the repertoire of signalling events that contribute to these responses. As such, another computational method was developed that could quantify the relative contributions of six different effectors to each ligand impedance response. In this study, we performed a log-time transformation of the impedance data that permitted responses to be resampled using a set of basis functions. Ultimately, this allowed each impedance response obtained to be represented by a set of seven individual factors. By calculating the cosine distance between the vectors generated from these seven factors for control and pathway-inhibitor pre-treatment conditions, we could represent the contribution of a given effector to a ligand's impedance response with a single descriptor. By generating descriptors for each of the effectors assessed, impedance profiles could be obtained for each ligand and its signalling profile could be visualized using star plots (Article 3, Figure 5, page 172). Compounds were then clustered by these star plots to obtain eleven distinct compound classes. Prototypical compounds from each of the compound classes predicted were subsequently assessed for their ability to modulate human cardiomyocyte contraction. Nine of the eleven compound classes were found to elicit distinct effects on the contractile response as determined by differences in the beat rate, amplitude and/or shape, demonstrating the ability of the computational approach to accurately predict functional distinctions *in vivo* from the impedance data obtained in an experimental cell line. Thus, this represents a powerful approach to assess and visualize ligand functional selectivity among a compound library, and may prove to be a valuable tool in the context of drug

discovery to identify functionally distinct compounds to be validated and characterized in further pre-clinical studies.

Being amenable to use with primary cells, label-free techniques also offer another advantage over conventional techniques to assay ligand pharmacological activity. While these cell types are often a challenge to adapt to conventional assays that require genetic manipulation (e.g. transfection of a biosensor), the non-invasive nature of label-free techniques provides a suitable assay platform to assess the effect of ligand treatment in the receptor's native context (see Article 1, Figure 8, page 101). In addition, recent advancements in the production of cell lines derived from induced pluripotent stem cells (iPSCs) represents a technological breakthrough that could be of great value in drug discovery efforts, allowing ligand pharmacological activity to be studied in cell lines derived directly from human tissues natively expressing the receptor of interest. Furthermore, the ability to derive and expand cells directly from diseased human tissue would provide the most clinically relevant context to assess the therapeutic potential of drugs in the laboratory. In combining the use of iPSCs with impedance profiling, one could obtain signalling profiles of a collection of compounds in a highly relevant physiological context. Moreover, impedance could be used as measure of the dysfunction in the signalling systems of disease-derived cells. Upon treatment of normal and disease-derived iPSCs with a physiologically relevant stimulus, differences in the impedance responses generated could represent the disturbances in network structure related to the pathological mechanism underlying the disease. Under the assumption that restoring the impedance response generated in the disease-derived cells to that which is obtained in the normal cells might indicate a rescue from the pathological mechanism(s), compounds targeting a receptor thought to provide therapeutic benefit in the disease could be assessed for their ability to restore a normal impedance response. This approach could be particularly helpful in diseases where the precise molecular determinants have not been completely elucidated.

Another potential use of impedance in drug discovery uses the measurement of cell attachment (which determines the impedance signal) over longer periods of time to assess

the effects of compound treatment on cell proliferation or death. As described in the Introduction (*Section 7.2*, page 57), impedance has previously been used to screen for anti-mitotic compounds (Abassi et al., 2009) by measuring the loss of cell attachment that accompanies cell cycle arrest and apoptosis. The role of GPCRs in the development and treatment of cancers is an emerging area of study (Lappano & Maggiolini, 2011) and impedance provides a potential platform to both classify compounds based on their acute impedance signatures in relevant cancer cell lines and monitor their subsequent effects on cell proliferation and death in a single experiment.

Altogether, label-free techniques provide a number of valuable tools to assess GPCR signalling that could add value to current drug discovery approaches.

3. The influence of network architecture on GPCR signalling and pharmacology

Signalling pathways are often conceptualized as a linear propagation of events initiated upon receptor activation. However, these modules rarely exist in isolation and instead have been found to be highly interconnected with other effectors, susceptible to signalling cross-talk and subject to a variety of feedback systems that have evolved to ensure an appropriate cellular response to external stimuli (Blüthgen & Legewie, 2013; Brandman & Meyer, 2008). Because of these properties, the use of reductionist approaches to classify ligands by their ability to modulate the activity of individual effectors could lead to a misrepresentation of their actual signalling capacities. In some cases, one pathway can influence the activation state of another pathway, and many of the canonical GPCR signalling events (e.g. production of second messengers, activation of kinase cascades, etc.) can both influence and be influenced by other signalling events. For example, increases in intracellular Ca^{2+} can modulate the production of cAMP through Ca^{2+}-sensitive adenylyl cyclase isoforms that are either activated (isoforms III, V, VIII) or inhibited (isoforms I and VI) upon Ca^{2+}/calmodulin binding (Halls & Cooper, 2011). Conversely, cAMP itself can potentiate the release of Ca^{2+} from intracellular stores in the endoplasmic reticulum, through a PKA-dependent phosphorylation of IP_3 receptors (Bruce, Shuttleworth, Giovannucci, & Yule, 2002). In addition, Ca^{2+} and cAMP have both been observed to

modulate activation of the ERK1/2 MAPK cascade at multiple levels (Gerits, Kostenko, Shiryaev, Johannessen, & Moens, 2008; Rozengurt, 2007). Thus the production/activation of these important signalling effectors cannot be considered truly independent from each other in many cases, as increasing the production of one can control the production or activation of another. Furthermore, the crosstalk observed between various GPCR signalling systems can have important implications for the cellular responses that are regulated by these pathways. For example, a highly coordinated integration of cAMP, MAPK and Ca^{2+} pathways has been implicated in hippocampus-dependent long term memory formation through convergence on calcium and cAMP response element (CRE)-dependent transcription (Figure 7; Xia and Storm 2012). Therefore, measurements of the activation state of these individual effectors may not correlate directly with the effects of ligands on the relevant physiological response to receptor stimulation.

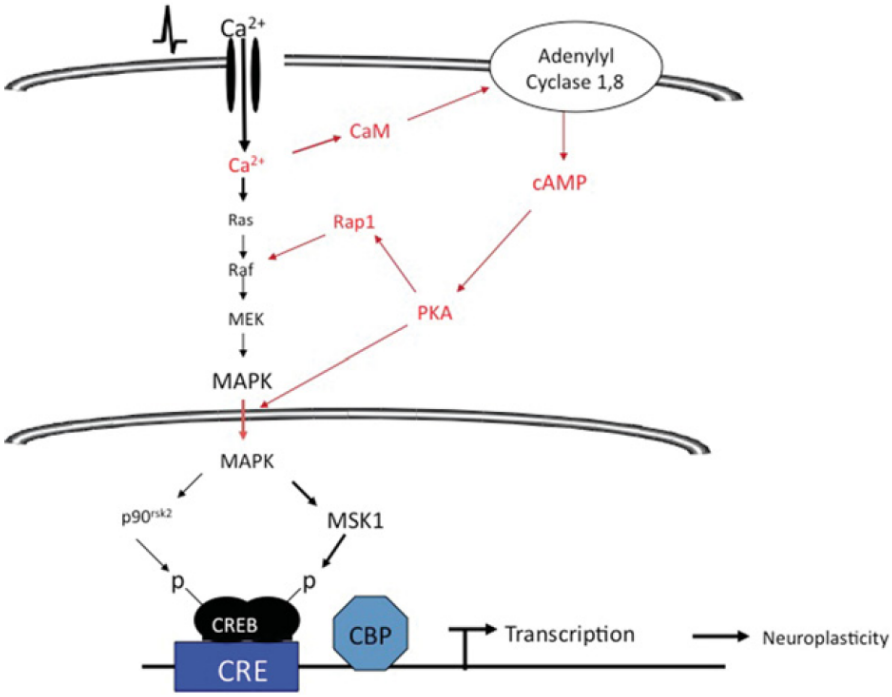


Figure 7: Integration of cAMP, MAPK and Ca^{2+} on CRE-dependent transcription
Increases in intracellular Ca^{2+} potentiates the production of cAMP by Ca^{2+}/calmodulin-sensitive adenylyl cyclases, which in turn both increases MAPK activation through the PKA effector Rap1 and MAPK translocation to the nucleus, to potentiate CRE-dependent transcription primarily through the MAPK effector MSK1. Adapted from Xia and Storm (2012)

In fact, in many cases it is not simply an additive function of the activation state of effectors that determines the magnitude of a higher-order cellular response. Both the ERK1/2 and Akt pathways are known to contribute to important cell fate decisions, such as the competition between continued cellular proliferation or differentiation. However, Chen et al. (2012) recently reported that sustained activation of neither effector correlated strongly with cellular proliferation, but rather that the relative activation states of both pathways determined the cellular fate. More specifically, a non-linear boundary on a two-dimensional plot of phospho-ERK1/2 versus phospho-Akt accurately predicted whether a cell would continue to proliferate or undergo neuronal differentiation (Figure 8). These results demonstrate that the significance of a given signalling event to an overall cellular response can be dependent on the greater signalling context of the cell. Often this signalling integration allows for important cellular responses to receive input from multiple external stimuli, ensuring a response that can be fine-tuned according to the precise physiological context.

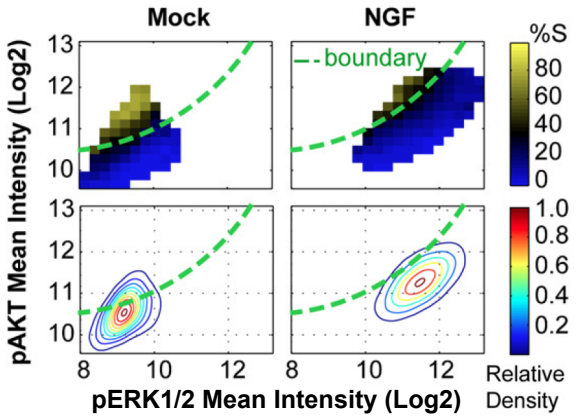


Figure 8: Influence of Akt and ERK1/2 pathways on cell proliferation and differentiation.

A heat map analysis of the role of pAkt-pERK1/2 signalling on cellular proliferation indicates a clear, non-linear boundary between conditions that promote proliferation or differentiation. On the top panels, proliferative cells are determined by the percent that are in S phase (%S). Bottom panel show contour plots indicating cell density. Treatment with nerve growth factor (NGF) does not change the boundary line between proliferative and differentiating cells, but instead shifts the population of cells towards differentiation through activation of both Akt and ERK1/2 pathways. Adapted from Chen et al. (2012)

Altogether, these examples demonstrate that (1) cross-talk between GPCR signalling pathways can create an interdependency among the signalling events engaged by a receptor, and (2) the importance of a given signalling event in the overall cellular response may not correlate directly with its level of activation and may be dependent on the activation state of other effectors converging on the response. These properties require that we conceptualize GPCR signalling not as a collection of independent linear pathways, but instead as a highly interconnected network of effectors, through which the emergence of a cellular response is determined by the overall network state. Thus, techniques that measure the changes in the overall signalling network underlying a given cellular response may provide a more appropriate readout of the pharmacological activity of a given ligand.

While label-free systems represent top-down approaches to assess how changes in the overall signalling network influence a higher-order cellular response, they do not provide a clear picture of how individual signalling events collaborate to produce this response. Techniques developed in the relatively new field of systems biology may provide the complementary bottom-up approach to reveal how GPCR signalling networks are structured and dynamically modulated upon receptor activation to produce the overall cellular response. While traditional biochemistry and molecular biology studies of cell signalling have focused on the individual components of signalling cascades, to determine their interaction partners and substrates, reaction rates and abundance in cells, systems approaches aim to synthesize this information into a holistic model of the signalling network and reveal how the connectivity and dynamics of these events determine a cellular response. While this approach has previously been used to study various signalling networks such as those responsive to mitogenic stimuli (Amit, Wides, & Yarden, 2007; Radisavljevic, 2013), controlling immunity (Zak & Aderem, 2009), metabolism (Metallo & Vander Heiden, 2013) and development (Tran, Lachke, & Stottmann, n.d.), or regulating the circadian clock (O'Neill, Maywood, & Hastings, 2013), researchers have yet to fully embrace these techniques in the study of GPCR signalling and its influence on cellular function. Although the yeast GPCR/MAPK pathways are a well established model system to study

how cells integrate input from multiple stimuli to elicit an appropriate cellular response using a systems approach (Kofahl & Klipp, 2004; McClean, Mody, Broach, & Ramanathan, 2007), an effort to extend these techniques into human physiology and pharmacology has yet to establish. While human systems are considerably more complex and challenging to study the precise molecular properties of a highly interconnected signalling network, a recent paper by Heitzler et al. (2012) exemplifies a systems-based approach to study GPCR signalling, combining both experimental data and computational modeling to describe the network architecture of $AT_{1A}R$-mediated ERK1/2 activation in human cells. In this study, researchers constructed a model of this highly interconnected signalling network by using prior knowledge of molecular interactions and populating it with experimentally determined effector concentrations and reaction rates. By comparing with the ERK1/2 activation kinetics observed, researchers employed an iterative process, testing different model structures, to ultimately arrive at a final dynamical model that matched extremely well with the experimentally derived data. This was not a purely an intellectual endeavour, however, as a novel antagonistic function of GRK2 on β-arrestin-dependent ERK1/2 activation was observed and experimentally validated.

The integrative nature of GPCR signalling networks has important consequences for exploiting ligand functional selectivity in drug discovery. By measuring and comparing the activation state of multiple effectors in a receptor's signalling network, as is commonly practiced, we can certainly reveal differences among ligands that reflect functional selectivity. Determining the biological relevance of these differences and translating the results obtained in experimental systems to *in vivo* conditions, however, remain formidable challenges. While the study by Heitzler et al. (2012) represents an exciting attempt to study GPCR signalling using a systems biology approach, because of the technical challenges to conduct such an analysis, it is limited to the assessment of a single signalling outcome (i.e. ERK1/2 activation) for only one ligand and does not consider the impact of this signalling network on a cell physiological response. The use of cellular impedance, however, allows us to probe for dynamic changes in the entire signalling network by measuring the overall cellular response to ligand treatment. Although impedance alone does not let us probe the precise dynamic molecular events that underlie a given cellular response, it does provide a

means to classify ligands by their effect on the entire signalling network. Instead, with the combined use of impedance and pathway-selective inhibitors (see Article 3), we can assess the *importance* of a given signalling event, as opposed to its *magnitude*, in generating a cellular response. For example, while two ligands might activate a given effector to the same extent, other differences in the signalling profiles of the ligands may influence the importance of that effector in the cellular response that is measured. With the combined use of impedance and pathway inhibitors we can detect such differences, while if we simply measured the activation state of the effector, both ligands would be classified the same. For example, in Article 1 the β_2AR ligands salbutamol and salmeterol were found to elicit very similar impedance responses, clustering into the same compound class and exhibiting similar effects on cAMP production and ERK1/2 activation (Article 1, Figure 4, page 94). However, with the use of pathway inhibitors to assess their impact on the overall β_2AR signalling network, additional functional differences were identified between ligands, resulting in their clustering into different compound classes (Article 3, Figure 4, page 169). G_i-dependent signalling, for example, was found to contribute to a much larger proportion of the salbumatol response compared to salmeterol (Article 3, Figure 2, page 167). Interestingly, while both of these compounds are used in the clinic to treat asthma, salmeterol has been found to be more effective and better tolerated than salbutamol for long term use (Boulet et al., 1997; Castle, Fuller, Hall, & Palmer, 1993). This is commonly attributed to the long-acting nature of salmeterol treatment, which is correlated with its relatively low efficacy to induce β_2AR endocytosis (Moore et al., 2007). However, it would be interesting to investigate whether the signalling differences detected by impedance profiling also contribute to its more desirable clinical profile.

Adapting this approach for use in normal or disease-derived iPS cells would allow the signalling networks to be assessed in a highly relevant cellular context. Given that differences in the expression of effectors can lead to dramatic changes the architecture and dynamics of signalling networks (Grecco, Schmick, & Bastiaens, 2011), the use of cell lines that most closely resemble the natural context of the receptor, either in normal physiology or disease, would provide the most reliable prediction of ligand activity in a given therapeutic context. In Article 1, we assessed whether the compound clustering obtained in

HEK293 cells was conserved in primary rat aortic smooth muscle cells endogenously expressing the receptor. Interestingly, the shapes of the impedance responses were completely different in aortic smooth muscle cells, with most ligands producing predominantly negative responses (Article 1, Figure 8, page 101), demonstrating the influence of cell type in the overall response to receptor activation. Despite these differences, ligands were found to cluster into very similar groups compared, with four out of five of the groups obtained in HEK293 cells eliciting similarly distinct impedance responses in aortic smooth muscle cells. These data suggest that despite differences in the cellular responses from cell type to cell type, clustering of compounds by their impedance signatures may incorporate a conserved, intrinsic property of ligand-receptor pairs that is representative of their fundamental pharmacological activity.

Thus, while systems biology techniques will undoubtedly revolutionize the study of GPCR signalling and pharmacology, label-free systems such as cellular impedance provide an alternative, yet complementary, approach to holistically assess GPCR signalling networks in the context of drug discovery. With continued development of both approaches, we may in the future witness a union of the two, with each approach informing the other to provide a highly descriptive and quantitative explanation of the mechanistic basis of drug action. Large data sets obtained using impedance combined with various pathway inhibitors could be parsed using the computational approaches developed for systems analyses, which could provide insight into the connectedness and inform the modeling of the signalling network. Hypotheses could then be generated and tested at both the level of the individual signalling components and on the overall cellular response in an iterative process, to ultimately provide an extensive description of the signalling network by combining both top-down and bottom-up approaches.

4. Receptor theory: Redux

With the wealth of evidence that has accumulated in the previous two decades, it has become difficult, if not impossible to reconcile the diversity of ligand activity at GPCRs into the previous models of GPCR activation. Several theorists have attempted to extend earlier models to incorporate additional active receptor states (Christopoulos & Kenakin, 2002;

Leff, Scaramellini, Law, & McKechnie, 1997). However, the general applicability of any model that defines a finite number of active receptor species, regardless of how numerous they might be, will remain uncertain awaiting a conclusive demonstration that receptor activity across the entire GPCR family proceeds through a fixed number of receptor conformations. Interestingly, the retinal GPCR rhodopsin, which is far more amenable to structural studies, does provide precedence for a fixed number of conformational states in its activation (Smith 2012). However, with the extraordinary range of receptor behaviours observed across the GPCR family when stimulated with ligands or upon mutation, it may be an exercise in futility to attempt to enumerate them. In Article 3 (Figure 5, page 171), for example, we present evidence for 11 functionally distinct classes of ligands at the β_2AR. Incorporating such observations into a single, cohesive receptor theory may instead require a fundamental change in our conception of the conformational basis GPCR signalling.

The spectroscopic and NMR studies outlined in the Introduction (*Section 5*, page 35) provide some insight into how receptors might couple to and exhibit selectivity for multiple distinct effectors. Many of these studies indicate that receptors can assume multiple conformations, even in the absence of ligand binding. These and other data accumulated from functional and MD simulation studies have suggested that receptors exist in a *conformational landscape*, populating multiple stable states at any given time (Kobilka & Deupi, 2007; Vaidehi & Kenakin, 2010). This dynamic model of receptor activation proposes that receptors exist in a continuum of conformational states, each with its own specific abilities to activate (or not) effectors (Figure 9). The fraction of receptors occupying any given state would be determined by the total energy of that state. Certain conformations with lower energy would be more favourable and thus more highly populated. The level of constitutive activity of a receptor would be explained by the energy barrier between the inactive and active signalling state(s) (Figure 9A) of the receptor in the absence of a ligand. Ligand binding would alter this energy landscape by stabilizing certain intramolecular interactions in the receptor at the cost of others, leading to a change in the distribution of conformations. Full agonists of G protein-dependent pathways, for example, would selectively stabilize the active conformation of the receptor that couples to G

proteins (Figure 9C; R*), reducing its total energy while increasing the energy of other inactive or intermediate states, while an inverse agonist would reduce the energy of the inactive, non-G protein-coupled state (Figure 9B; R''). Partial agonism of a given signalling response could be attained through several, non-mutually exclusive mechanisms. They could (1) stabilize intermediate states with less activity toward the effector, (2) exhibit less selectivity between the active and inactive states for the effector compared to full agonists or (3) induce slightly different conformational changes than full agonists that result in receptor conformations with decreased activity to activate the effector (Figure 9D; R^*_2). Ligand functional selectivity could therefore be explained by *conformational selection* of various receptor states with different intrinsic signalling activities towards effectors. Slight differences in the conformational landscapes stabilized by different compounds could give rise to exceptional signalling diversity, such as what is presented in Article 3, with the observation of eleven functionally distinct compound classes.

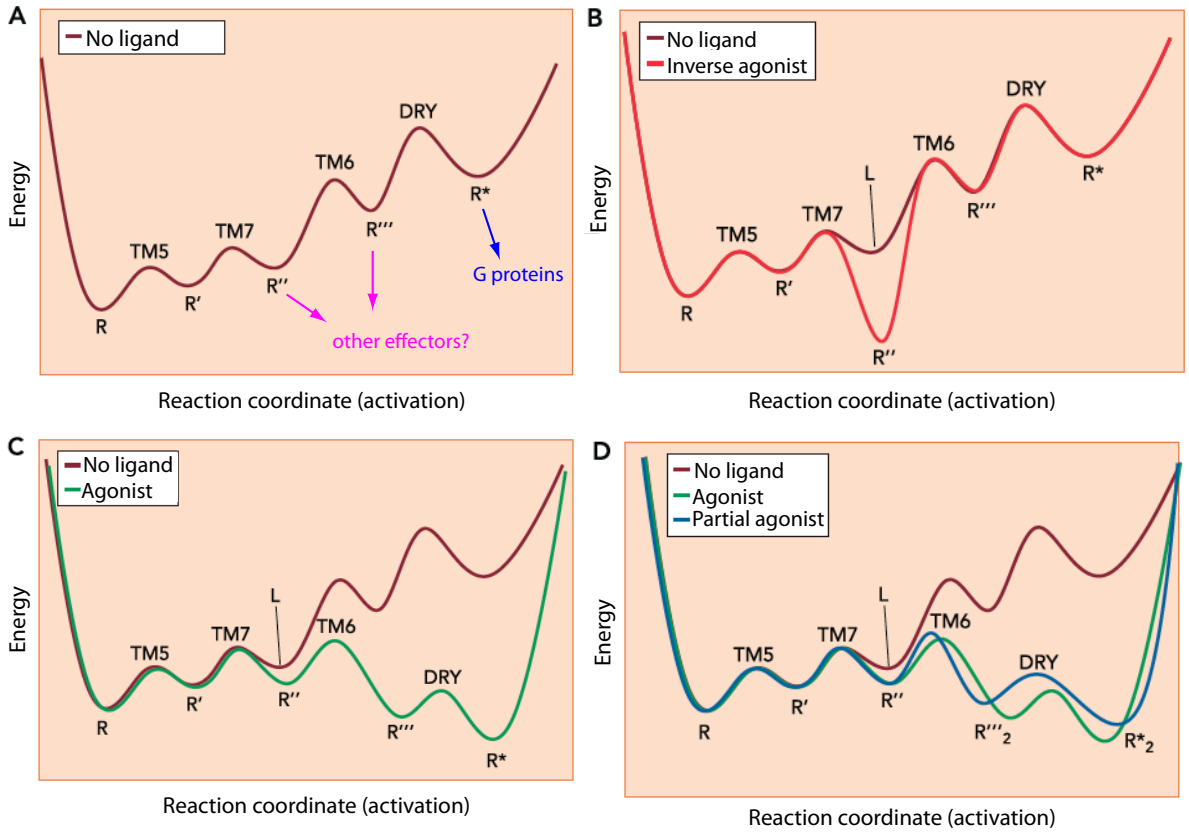


Figure 9: Conformational landscapes of GPCR activation with ligands of different efficacies.
Receptors exist in a conformational equilibrium between several pseudo-stable states where interconversion depends on the energy barrier between two adjacent states. Overcoming known conformational events in receptor activation, such as changes in the TM3/TM5 interface, rearrangement of the NPxxY motif in TM7, movement of TM6 and breaking of the ionic lock (DRY motif) may represent the energy barriers separating each receptor state. To explain ligand efficacy towards a single signalling event, such as G protein activation, we simply need to consider the transition of the receptor to an active state (R*) that couples to G proteins. (A) In the absence of ligand, the receptor exists in multiple conformations populated in accordance with the energy of each state. The level of constitutive signalling activity of the unliganded receptor would reflect the energy barrier between the inactive and active state (R*) of the receptor. (B) Binding of inverse agonists could stabilize a conformation of receptor that does not engage G proteins (R'') by lowering its total energy. (C) Similarly, binding of an agonist would reduce the energy require to assume the active signalling state of the receptor (R*). (D) Partial agonists, on the other hand, may lower the energy of active receptor states but stabilize conformations with reduced activity toward G protein activation (R^*_2). This model could be extended to account for multiple signalling events engaged by the receptor if we propose that intermediate states (e.g. R'', R''') might themselves have activity towards other effectors. Adapted from Deupi and Kobilka (2010).

At this level, however, the conformational landscape model does not provide a more complete description of receptor activity than previously models, such at the CTC model. In fact, the CTC model is far more operational since the relative populations of the receptor states it proposes and their rates of interconversion can more easily be obtained experimentally. The main advantage of the conformational landscape model is its ability to incorporate multiple effector coupling and ligand functional selectivity effectively into a single model of receptor activation. The simplest way to explain these phenomena using the 2-D energy landscapes presented in Figure 8 would be that the individual low energy states of the receptor each have their own intrinsic abilities to couple to various effectors and elicit a cellular response. The proportion of receptors occupying these various signalling states would therefore determine the signalling profile of a ligand. The validity of such a model requires that receptors are limited to a linear conformational pathway between individual states; that conformational changes always progress through the same

set of intramolecular rearrangements between two extreme conformations that delineate the full conformational potential of a given receptor (Figure 9). An alternative model of the conformational landscape allows for some divergence in the conformational pathways accessible to a receptor. As conformational changes propagate through a receptor, with certain intramolecular interactions forming and others breaking, a point of divergence might be encountered where multiple subsequent conformational rearrangements are accessible energetically. The precise conformational changes that occur at this "decision point" will be subject to probability based on the relative energies of the possible rearrangements, allowing the receptor to proceed down one of several parallel, yet divergent conformational pathways that unlock different subsequent conformational states of the receptor. In addition, pre-coupling with different effectors could significantly influence the conformational pathways accessible to a receptor, biasing specific proportions of the receptor towards a given conformational state. This model (as shown in Figure 10) vastly increases the conformational potential of a receptor, permitting multiple conformational pathways and leading to the stabilization of various structurally distinct conformations with differing abilities to couple to effectors. A ligand, therefore, may exhibit a selectivity for the conformational pathways taken by receptor resulting in vastly different conformational landscapes. The extent to which this is possible is not clear, however, several recent biophysical studies have indeed indicated that biased ligands can promote distinct conformational changes compared to unbiased compounds (Rahmeh et al. 2012; Liu et al. 2012). Undoubtedly, further technological innovations are required to assess the accuracy of such a model, likely through the continued development of biophysical techniques such as NMR that could provide high-resolution details of the receptor conformational dynamics and energetics underlying this mechanism of receptor activity.

With the continued development of technologies to probe both the structural determinants of GPCR signalling with greater resolution and reveal the vast functional diversity of GPCR signalling networks, we will continue to improve our conceptualization of GPCR function, incorporating new insights into increasingly elegant models of receptor activity.

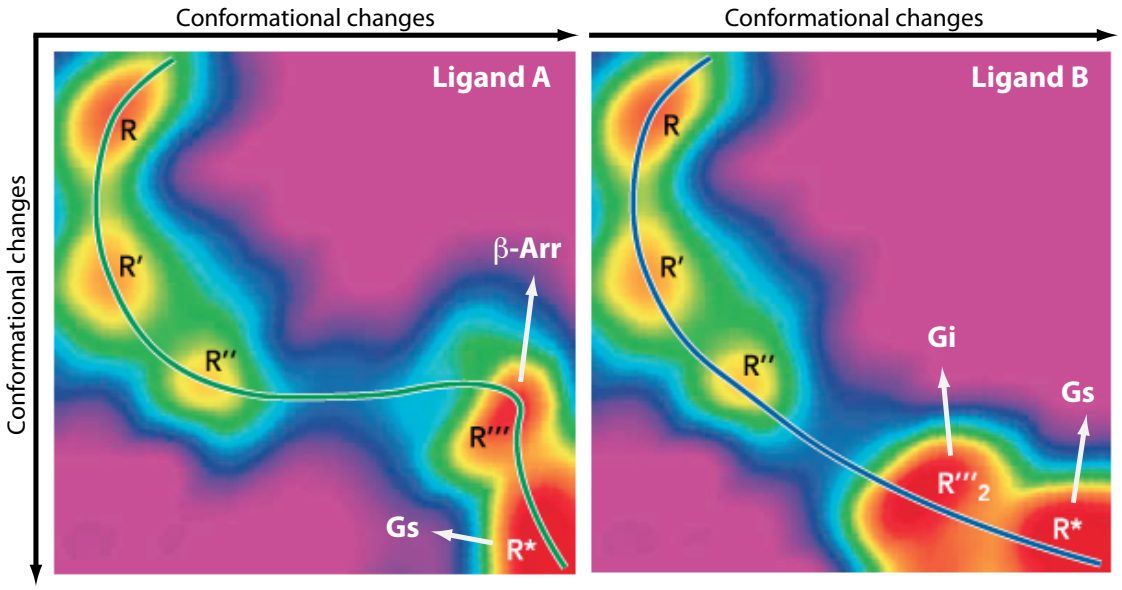


Figure 10: Divergent conformational pathways in receptor activation.
This theoretical model depicts distinct conformational changes (represented by position on x- and y-axes) induced upon binding of two functionally selective ligands. The relative population (or energy) of each state is represented by the z-axis colour (red: most highly populated, to magenta: least highly populated). The conformational pathway taken by each ligand is indicated by the path traced through the 3-D energy landscape. Both Ligands A and B induce conformational changes that ultimately lead to the stabilization of an active state (R*) that preferentially couples to G_s. Ligand A, however, induces a conformational change when transitioning from R'' to adopt a conformation (R''') that preferentially couples to β-arrestin (β-Arr), while Ligand B induces a different conformational change that transitions through a receptor conformation that preferentially couples to G_i (R'''_2). Adapted from Deupi and Kobilka (2010)

CONCLUSION

The work presented in this thesis demonstrates how label-free techniques such as cellular impedance can be used to assess ligand functional selectivity at GPCRs.

Impedance signatures are a composite representation of the signalling pathways engaged following receptor stimulation. Indeed, with the use of pharmacological inhibitors targeting key pathways of the β_2AR signalling repertoire, several of the canonical pathways were confirmed to contribute to the impedance response generated by the prototypical agonist isoproterenol. Functionally selective ligands were found to elicit distinct impedance signatures in a screen of β_2AR ligands, which correlated strongly with the signalling profiles of the ligands measured using traditional endpoint assays. With the development of novel computational methods, ligands could be clustered by their impedance signatures, revealing five distinct functional classes within the compound library and demonstrating more signalling texture than previously recognized for the receptor.

In addition, because impedance provides a holistic assessment of ligand signalling activity, the influence of pathways that are unknown to couple to the receptor are integrated into the impedance response generated by a ligand. In the process of dissecting the isoproterenol impedance response, a novel Ca^{2+} pathway was identified to contribute to the overall cellular response to β_2AR stimulation. Through a mechanistic determination of this response, it was revealed that the β_2AR induces a G_s-dependent transactivation of P2Y11 purinergic receptors. These results could contribute to our understanding of the interplay between adrenergic and purinergic signalling systems in physiology and potentially impact future drug discovery efforts for pulmonary diseases such as asthma, in which these systems contribute to the pathophysiological mechanism and its treatment.

In an extension of the screening approach developed, cellular impedance was combined with the use of pathway-selective inhibitors to provide even greater resolution of the functional differences among ligands. Another novel computational approach was developed that could measure the contribution of six pathways to a ligand's impedance signature. This approach allowed ligand signalling profiles to be assessed, visualized and

clustered from the data obtained. Applying this approach to an uncharacterized library of compounds, novel functional classes were observed. Compounds belonging to different compound classes were found to have distinct effects on human cardiomyocyte contractility, validating the functional differences observed by impedance profiling.

Given the prevalence of ligand functional selectivity and its importance in both physiological and therapeutic contexts, approaches that are capable of assessing the full signalling spectrum of drugs represent important tools in the experimental arsenal of GPCR scientists. Based on its ability to functionally distinguish compounds based on their overall effect on a cellular response, the addition of impedance profiling to current drug discovery strategies could lead to the identification of safer and more efficacious therapeutic treatments of human disease.

REFERENCES

Abassi, Y. A., Xi, B., Zhang, W., Ye, P., Kirstein, S. L., Gaylord, M. R., … Xu, X. (2009). Kinetic cell-based morphological screening: prediction of mechanism of compound action and off-target effects. *Chemistry & biology*, *16*(7), 712–23. doi:10.1016/j.chembiol.2009.05.011

Abel, A., Wittau, N., Wieland, T., Schultz, G., & Kalkbrenner, F. (2000). Cell cycle-dependent coupling of the vasopressin V1a receptor to different G proteins. *The Journal of biological chemistry*, *275*(42), 32543–51. doi:10.1074/jbc.M002171200

Abramson, S. N., Martin, M. W., Hughes, A. R., Harden, T. K., Neve, K. A., Barrett, D. A., & Molinoff, P. B. (1988). Interaction of beta-adrenergic receptors with the inhibitory guanine nucleotide-binding protein of adenylate cyclase in membranes prepared from cyc- S49 lymphoma cells. *Biochemical pharmacology*, *37*(22), 4289–97.

Ahn, S. (1999). Src-mediated Tyrosine Phosphorylation of Dynamin Is Required for beta 2-Adrenergic Receptor Internalization and Mitogen-activated Protein Kinase Signaling. *Journal of Biological Chemistry*, *274*(3), 1185–1188. doi:10.1074/jbc.274.3.1185

Ahrén, B. (2009). Islet G protein-coupled receptors as potential targets for treatment of type 2 diabetes. *Nature reviews. Drug discovery*, *8*(5), 369–85. doi:10.1038/nrd2782

Akam, E. C., Challiss, R. A., & Nahorski, S. R. (2001). G(q/11) and G(i/o) activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subtype. *British journal of pharmacology*, *132*(4), 950–8. doi:10.1038/sj.bjp.0703892

Akinaga, J., Lima, V., Kiguti, L. R. de A., Hebeler-Barbosa, F., Alcantara-Hernandez, R., Garcia-Sainz, J. A., & Pupo, A. S. (2013). Differential Phosphorylation, Desensitization and Internalization of α1A Adrenoceptors Activated by Norepinephrine and Oxymetazoline. *Molecular pharmacology*. doi:10.1124/mol.112.082313

Al-Ahmadi, W., Al-Haj, L., Al-Mohanna, F. A., Silverman, R. H., & Khabar, K. S. A. (2009). RNase L downmodulation of the RNA-binding protein, HuR, and cellular growth. *Oncogene*, *28*(15), 1782–91. doi:10.1038/onc.2009.16

Allgeier, A., Laugwitz, K. L., Van Sande, J., Schultz, G., & Dumont, J. E. (1997). Multiple G-protein coupling of the dog thyrotropin receptor. *Molecular and cellular endocrinology*, *127*(1), 81–90.

Allgeier, A., Offermanns, S., Van Sande, J., Spicher, K., Schultz, G., & Dumont, J. E. (1994). The human thyrotropin receptor activates G-proteins Gs and Gq/11. *The Journal of biological chemistry*, *269*(19), 13733–5.

Altier, C. (2012). GPCR and Voltage-Gated Calcium Channels (VGCC) Signaling Complexes. In *GPCR Signalling Complexes – Synthesis, Assembly, Trafficking and Specificity* (Vol. 63, pp. 241–62). doi:10.1007/978-94-007-4765-4_13

Alvarez, S. E., Seguin, L. R., Villarreal, R. S., Nahmias, C., & Ciuffo, G. M. (2008). Involvement of c-Src tyrosine kinase in SHP-1 phosphatase activation by Ang II AT2 receptors in rat fetal tissues. *Journal of cellular biochemistry*, *105*(3), 703–11. doi:10.1002/jcb.21866

Amit, I., Wides, R., & Yarden, Y. (2007). Evolvable signaling networks of receptor tyrosine kinases: relevance of robustness to malignancy and to cancer therapy. *Molecular systems biology*, *3*, 151. doi:10.1038/msb4100195

Arey, B. J. (1997). Induction of Promiscuous G Protein Coupling of the Follicle-Stimulating Hormone (FSH) Receptor: A Novel Mechanism for Transducing Pleiotropic Actions of FSH Isoforms. *Molecular Endocrinology*, *11*(5), 517–526. doi:10.1210/me.11.5.517

Ariëns, E. J. (1954). Affinity and intrinsic activity in the theory of competitive inhibition. I. Problems and theory. *Archives internationales de pharmacodynamie et de thérapie*, *99*(1), 32–49.

Attramadal, H., Arriza, J. L., Aoki, C., Dawson, T. M., Codina, J., Kwatra, M. M., … Lefkowitz, R. J. (1992). Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *The Journal of biological chemistry*, *267*(25), 17882–90.

Audet, M., & Bouvier, M. (2012). Restructuring G-protein- coupled receptor activation. *Cell*, *151*(1), 14–23. doi:10.1016/j.cell.2012.09.003

Audet, N., Galés, C., Archer-Lahlou, E., Vallières, M., Schiller, P. W., Bouvier, M., & Pineyro, G. (2008). Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing delta-opioid receptors and heterotrimeric G proteins. *The Journal of biological chemistry*, *283*(22), 15078–88. doi:10.1074/jbc.M707941200

Audet, N., Paquin-Gobeil, M., Landry-Paquet, O., Schiller, P. W., & Piñeyro, G. (2005). Internalization and Src activity regulate the time course of ERK activation by delta opioid receptor ligands. *The Journal of biological chemistry*, *280*(9), 7808–16. doi:10.1074/jbc.M411695200

Azzi, M., Charest, P. G., Angers, S., Rousseau, G., Kohout, T., Bouvier, M., & Piñeyro, G. (2003). Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(20), 11406–11. doi:10.1073/pnas.1936664100

Azzi, M., Pineyro, G., Pontier, S., Parent, S., Ansanay, H., & Bouvier, M. (2001). Allosteric Effects of G Protein Overexpression on the Binding of beta -Adrenergic Ligands with Distinct Inverse Efficacies. *Mol. Pharmacol.*, *60*(5), 999–1007.

Bailey, C. P., Kelly, E., & Henderson, G. (2004). Protein kinase C activation enhances morphine-induced rapid desensitization of mu-opioid receptors in mature rat locus ceruleus neurons. *Molecular pharmacology*, *66*(6), 1592–8. doi:10.1124/mol.104.004747

Baillie, G. S., Sood, A., McPhee, I., Gall, I., Perry, S. J., Lefkowitz, R. J., & Houslay, M. D. (2005). Beta-arrestin-recruited phosphodiesterase-4 desensitizes the AKAP79/PKA-mediated switching of beta2-adrenoceptor signalling to activation of ERK. *Biochemical Society transactions*, *33*(Pt 6), 1333–6. doi:10.1042/BST20051333

Ballesteros, J A, Jensen, A. D., Liapakis, G., Rasmussen, S. G., Shi, L., Gether, U., & Javitch, J. A. (2001). Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *The Journal of biological chemistry*, *276*(31), 29171–7. doi:10.1074/jbc.M103747200

Ballesteros, Juan A., & Weinstein, H. (1995). Receptor Molecular Biology. *Methods in Neurosciences*, *25*(366-428). doi:10.1016/S1043-9471(05)80049-7

Barlic, J., Andrews, J. D., Kelvin, A. A., Bosinger, S. E., DeVries, M. E., Xu, L., … Kelvin, D. J. (2000). Regulation of tyrosine kinase activation and granule release through beta-arrestin by CXCRI. *Nature immunology*, *1*(3), 227–33. doi:10.1038/79767

Barnes, P. J. (2011). Biochemical basis of asthma therapy. *The Journal of biological chemistry*, *286*(38), 32899–905. doi:10.1074/jbc.R110.206466

Barnes, W. G., Reiter, E., Violin, J. D., Ren, X.-R., Milligan, G., & Lefkowitz, R. J. (2005). beta-Arrestin 1 and Galphaq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation. *The Journal of biological chemistry*, *280*(9), 8041–50. doi:10.1074/jbc.M412924200

Barr, A. J., Brass, L. F., & Manning, D. R. (1997). Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. A direct evaluation of selectivity in receptor.G protein coupling. *The Journal of biological chemistry*, *272*(4), 2223–9.

Beaulieu, J.-M., Marion, S., Rodriguiz, R. M., Medvedev, I. O., Sotnikova, T. D., Ghisi, V., … Caron, M. G. (2008). A beta-arrestin 2 signaling complex mediates lithium action on behavior. *Cell*, *132*(1), 125–36. doi:10.1016/j.cell.2007.11.041

Beaulieu, J.-M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R., & Caron, M. G. (2005). An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell*, *122*(2), 261–73. doi:10.1016/j.cell.2005.05.012

Beedle, A. M., McRory, J. E., Poirot, O., Doering, C. J., Altier, C., Barrere, C., … Zamponi, G. W. (2004). Agonist-independent modulation of N-type calcium channels by ORL1 receptors. *Nature neuroscience*, *7*(2), 118–25. doi:10.1038/nn1180

Benovic, J. L., Kühn, H., Weyand, I., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1987). Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein). *Proceedings of the National Academy of Sciences of the United States of America*, *84*(24), 8879–82.

Benovic, J. L., Pike, L. J., Cerione, R. A., Staniszewski, C., Yoshimasa, T., Codina, J., … Lefkowitz, R. J. (1985). Phosphorylation of the mammalian beta-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guan. *The Journal of biological chemistry*, *260*(11), 7094–101.

Berg, K., Maayani, S., Goldfarb, J., Scaramellini, C., Leff, P., & Clarke, W. P. (1998). Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Molecular pharmacology*, *54*(1), 94–104.

Bernstein, L. S., Ramineni, S., Hague, C., Cladman, W., Chidiac, P., Levey, A. I., & Hepler, J. R. (2004). RGS2 binds directly and selectively to the M1 muscarinic acetylcholine receptor third intracellular loop to modulate Gq/11alpha signaling. *The Journal of biological chemistry*, *279*(20), 21248–56. doi:10.1074/jbc.M312407200

Berrada, K., Plesnicher, C. L., Luo, X., & Thibonnier, M. (2000). Dynamic interaction of human vasopressin/oxytocin receptor subtypes with G protein-coupled receptor kinases and protein kinase C after agonist stimulation. *The Journal of biological chemistry*, *275*(35), 27229–37. doi:10.1074/jbc.M002288200

Bisello, A., Chorev, M., Rosenblatt, M., Monticelli, L., Mierke, D. F., & Ferrari, S. L. (2002). Selective ligand-induced stabilization of active and desensitized parathyroid hormone type 1 receptor conformations. *The Journal of biological chemistry*, *277*(41), 38524–30. doi:10.1074/jbc.M202544200

Black, J. W. (1988). Drugs from emasculated hormones: The principles of synoptic antagonisms.

BLACK, J. W., FISHER, E. W., & SMITH, A. N. (1958). The effects of 5-hydroxytryptamine on gastric secretion in anaesthetized dogs. *The Journal of physiology*, *141*(1), 27–34.

Black, J. W., & Leff, P. (1983). Operational models of pharmacological agonism. *Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character. Royal Society (Great Britain)*, *220*(1219), 141–62.

Blüthgen, N., & Legewie, S. (2013). Robustness of signal transduction pathways. *Cellular and molecular life sciences : CMLS*, *70*(13), 2259–69. doi:10.1007/s00018-012-1162-7

Bockaert, J., Fagni, L., Dumuis, A., & Marin, P. (2004). GPCR interacting proteins (GIP). *Pharmacology & therapeutics*, *103*(3), 203–21. doi:10.1016/j.pharmthera.2004.06.004

Boerrigter, G., Lark, M. W., Whalen, E. J., Soergel, D. G., Violin, J. D., & Burnett, J. C. (2011). Cardiorenal actions of TRV120027, a novel ß-arrestin-biased ligand at the angiotensin II type I receptor, in healthy and heart failure canines: a novel therapeutic strategy for acute heart failure. *Circulation. Heart failure*, *4*(6), 770–8. doi:10.1161/CIRCHEARTFAILURE.111.962571

Bohn, L M, Dykstra, L. A., Lefkowitz, R. J., Caron, M. G., & Barak, L. S. (2004). Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. *Molecular pharmacology*, *66*(1), 106–12. doi:10.1124/mol.66.1.106

Bohn, Laura M., Gainetdinov, R. R., Sotnikova, T. D., Medvedev, I. O., Lefkowitz, R. J., Dykstra, L. A., & Caron, M. G. (2003). Enhanced Rewarding Properties of Morphine, but not Cocaine, in {beta}arrestin-2 Knock-Out Mice. *J. Neurosci.*, *23*(32), 10265–10273.

Bokoch, M. P., Zou, Y., Rasmussen, S. G. F., Liu, C. W., Nygaard, R., Rosenbaum, D. M., … Kobilka, B. K. (2010). Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature*, *463*(7277), 108–12. doi:10.1038/nature08650

Bonhaus, D. W., Chang, L. K., Kwan, J., & Martin, G. R. (1998). Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for agonist-specific trafficking of intracellular responses. *The Journal of pharmacology and experimental therapeutics*, *287*(3), 884–8.

Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., … Casellas, P. (1997). A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *The Journal of biological chemistry*, *272*(35), 22330–9.

Boudin, H., Doan, A., Xia, J., Shigemoto, R., Huganir, R. L., Worley, P., & Craig, A. M. (2000). Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. *Neuron*, *28*(2), 485–97.

Boulet, L. P., Laviolette, M., Boucher, S., Knight, A., Hébert, J., & Chapman, K. R. (1997). A twelve-week comparison of salmeterol and salbutamol in the treatment of mild-to-moderate asthma: a Canadian multicenter study. *The Journal of allergy and clinical immunology*, *99*(1 Pt 1), 13–21.

Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B. F., Kobilka, B. K., Caron, M. G., & Lefkowitz, R. J. (1988). Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature*, *333*(6171), 370–3. doi:10.1038/333370a0

Bouvier, M., Leeb-Lundberg, L. M., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1987). Regulation of adrenergic receptor function by phosphorylation. II. Effects of agonist occupancy on phosphorylation of alpha 1- and beta 2-adrenergic receptors by protein kinase C and the cyclic AMP-dependent protein kinase. *The Journal of biological chemistry*, *262*(7), 3106–13.

Brandman, O., & Meyer, T. (2008). Feedback loops shape cellular signals in space and time. *Science (New York, N.Y.)*, *322*(5900), 390–5. doi:10.1126/science.1160617

Breit, A., Gagnidze, K., Devi, L. A., Lagacé, M., & Bouvier, M. (2006). Simultaneous activation of the delta opioid receptor (deltaOR)/sensory neuron-specific receptor-4 (SNSR-4) hetero-oligomer by the mixed bivalent agonist bovine adrenal medulla peptide 22 activates SNSR-4 but inhibits deltaOR signaling. *Molecular pharmacology*, *70*(2), 686–96. doi:10.1124/mol.106.022897

Breton, B., Lagacé, M., & Bouvier, M. (2010). Combining resonance energy transfer methods reveals a complex between the {alpha}2A-adrenergic receptor, G{alpha}i1{beta}1{gamma}2, and GRK2. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *24*, 1–11. doi:10.1096/fj.10-164061

Brinks, H. L., & Eckhart, A. D. (2010). Regulation of GPCR signaling in hypertension. *Biochimica et biophysica acta*, *1802*(12), 1268–75. doi:10.1016/j.bbadis.2010.01.005

Brown, G. P., & Pasternak, G. W. (1998). 3H-naloxone benzoylhydrazone binding in MOR-1-transfected Chinese hamster ovary cells: evidence for G-protein-dependent antagonist binding. *The Journal of pharmacology and experimental therapeutics*, *286*(1), 376–81.

Bruce, J. I. E., Shuttleworth, T. J., Giovannucci, D. R., & Yule, D. I. (2002). Phosphorylation of inositol 1,4,5-trisphosphate receptors in parotid acinar cells. A mechanism for the synergistic effects of cAMP on Ca2+ signaling. *The Journal of biological chemistry*, *277*(2), 1340–8. doi:10.1074/jbc.M106609200

Brydon, L., Roka, F., Petit, L., de Coppet, P., Tissot, M., Barrett, P., … Jockers, R. (1999). Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins. *Molecular endocrinology (Baltimore, Md.)*, *13*(12), 2025–38.

Cali, J. J., Zwaagstra, J. C., Mons, N., Cooper, D. M., & Krupinski, J. (1994). Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. *The Journal of biological chemistry*, *269*(16), 12190–5.

Cao, W., Luttrell, L. M., Medvedev, A. V, Pierce, K. L., Daniel, K. W., Dixon, T. M., … Collins, S. (2000). Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. *The Journal of biological chemistry*, *275*(49), 38131–4. doi:10.1074/jbc.C000592200

Capuano, B., Crosby, I. T., & Lloyd, E. J. (2002). Schizophrenia: genesis, receptorology and current therapeutics. *Current medicinal chemistry*, *9*(5), 521–48.

Carman, C. V. (1999). Selective Regulation of Galpha q/11 by an RGS Domain in the G Protein-coupled Receptor Kinase, GRK2. *Journal of Biological Chemistry*, *274*(48), 34483–34492. doi:10.1074/jbc.274.48.34483

Castle, W., Fuller, R., Hall, J., & Palmer, J. (1993). Serevent nationwide surveillance study: comparison of salmeterol with salbutamol in asthmatic patients who require regular bronchodilator treatment. *BMJ (Clinical research ed.)*, *306*(6884), 1034–7.

Catapano, L. A., & Manji, H. K. (2007). G protein-coupled receptors in major psychiatric disorders. *Biochimica et biophysica acta*, *1768*(4), 976–93. doi:10.1016/j.bbamem.2006.09.025

Chakrabarti, S., Prather, P. L., Yu, L., Law, P. Y., & Loh, H. H. (1995). Expression of the mu-opioid receptor in CHO cells: ability of mu-opioid ligands to promote alpha-azidoanilido[32P]GTP labeling of multiple G protein alpha subunits. *Journal of neurochemistry*, *64*(6), 2534–43.

Charest, P. G., Terrillon, S., & Bouvier, M. (2005). Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. *EMBO reports*, *6*(4), 334–40. doi:10.1038/sj.embor.7400373

Charlton, J. J., Allen, P. B., Psifogeorgou, K., Chakravarty, S., Gomes, I., Neve, R. L., … Zachariou, V. (2008). Multiple actions of spinophilin regulate mu opioid receptor function. *Neuron*, *58*(2), 238–47. doi:10.1016/j.neuron.2008.02.006

Chen, J.-Y., Lin, J.-R., Cimprich, K. A., & Meyer, T. (2012). A two-dimensional ERK-AKT signaling code for an NGF-triggered cell-fate decision. *Molecular cell*, *45*(2), 196–209. doi:10.1016/j.molcel.2011.11.023

Cherezov, V., Rosenbaum, D. M., Hanson, M. a, Rasmussen, S. G. F., Thian, F. S., Kobilka, T. S., … Stevens, R. C. (2007). High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science (New York, N.Y.)*, *318*(5854), 1258–65. doi:10.1126/science.1150577

Chidiac, P., Hebert, T. E., Valiquette, M., Dennis, M., & Bouvier, M. (1994). Inverse agonist activity of beta-adrenergic antagonists. *Molecular pharmacology*, *45*(3), 490–9.

Chien, E. Y. T., Liu, W., Zhao, Q., Katritch, V., Won Han, G., Hanson, M. a., … Stevens, R. C. (2010). Structure of the Human Dopamine D3 Receptor in Complex with a D2/D3 Selective Antagonist. *Science*, *330*(6007), 1091–1095. doi:10.1126/science.1197410

Choi, E. J., Wong, S. T., Hinds, T. R., & Storm, D. R. (1992). Calcium and muscarinic agonist stimulation of type I adenylylcyclase in whole cells. *J. Biol. Chem.*, *267*(18), 12440–12442.

Christopoulos, A., & Kenakin, T. P. (2002). G Protein-Coupled Receptor Allosterism and Complexing. *Pharmacological Reviews*, *54*(2), 323–374. doi:10.1124/pr.54.2.323

Claing, A., Chen, W., Miller, W. E., Vitale, N., Moss, J., Premont, R. T., & Lefkowitz, R. J. (2001). beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis. *The Journal of biological chemistry*, *276*(45), 42509–13. doi:10.1074/jbc.M108399200

Clark, A. J. (1937). *General Pharmacology: Heffter's Handbuch d. exp. Pharmacology (Ergband 4)*. Springer.

Cordeaux, Y., Briddon, S. J., Megson, A. E., McDonnell, J., Dickenson, J. M., & Hill, S. J. (2000). Influence of receptor number on functional responses elicited by agonists acting at the human adenosine A(1) receptor: evidence for signaling pathway-dependent changes in agonist potency and relative intrinsic activity. *Molecular pharmacology*, *58*(5), 1075–84.

Cordeaux, Y., Nickolls, S. A., Flood, L. A., Graber, S. G., & Strange, P. G. (2001). Agonist regulation of D(2) dopamine receptor/G protein interaction. Evidence for agonist selection of G protein subtype. *The Journal of biological chemistry*, *276*(31), 28667–75. doi:10.1074/jbc.M008644200

Costa, T., & Herz, A. (1989). Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(19), 7321–5.

Costa, T., Ogino, Y., Munson, P. J., Onaran, H. O., & Rodbard, D. (1992). Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic

interpretation of negative antagonism and of receptor activity in the absence of ligand. *Molecular pharmacology*, *41*(3), 549–60.

Crawford, K. W., Frey, E. A., & Cote, T. E. (1992). Angiotensin II receptor recognized by DuP753 regulates two distinct guanine nucleotide-binding protein signaling pathways. *Molecular pharmacology*, *41*(1), 154–62.

Cussac, D., Newman-Tancredi, A., Duqueyroix, D., Pasteau, V., & Millan, M. J. (2002). Differential activation of Gq/11 and Gi(3) proteins at 5-hydroxytryptamine(2C) receptors revealed by antibody capture assays: influence of receptor reserve and relationship to agonist-directed trafficking. *Molecular pharmacology*, *62*(3), 578–89.

Daaka, Y., Luttrell, L. M., & Lefkowitz, R. J. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature*, *390*(6655), 88–91. doi:10.1038/36362

Danussi, C., Petrucco, A., Wassermann, B., Pivetta, E., Modica, T. M. E., Del Bel Belluz, L., … Spessotto, P. (2011). EMILIN1-α4/α9 integrin interaction inhibits dermal fibroblast and keratinocyte proliferation. *The Journal of cell biology*, *195*(1), 131–45. doi:10.1083/jcb.201008013

De Lean, A., Stadel, J. M., & Lefkowitz, R. J. (1980). A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *The Journal of biological chemistry*, *255*(15), 7108–17.

De Vries, L., Zheng, B., Fischer, T., Elenko, E., & Farquhar, M. G. (2000). The regulator of G protein signaling family. *Annual review of pharmacology and toxicology*, *40*, 235–71. doi:10.1146/annurev.pharmtox.40.1.235

DeFea, K. A., Vaughn, Z. D., O'Bryan, E. M., Nishijima, D., Déry, O., & Bunnett, N. W. (2000). The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(20), 11086–91. doi:10.1073/pnas.190276697

DeFea, K. A., Zalevsky, J., Thoma, M. S., Déry, O., Mullins, R. D., & Bunnett, N. W. (2000). beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *The Journal of cell biology*, *148*(6), 1267–81.

DeMaria, S., & Ngai, J. (2010). The cell biology of smell. *The Journal of cell biology*, *191*(3), 443–52. doi:10.1083/jcb.201008163

Depry, C., Allen, M. D., & Zhang, J. (2010). Visualization of PKA activity in plasma membrane microdomains. *Molecular bioSystems*, *7*, 52–58. doi:10.1039/c0mb00079e

Deupi, X., & Kobilka, B. K. (2010). Energy Landscapes as a Tool to Integrate GPCR Structure, Dynamics, and Function. *Physiology (Bethesda, Md.)*, *25*(5), 293–303. doi:10.1152/physiol.00002.2010

DeWire, S. M., Ahn, S., Lefkowitz, R. J., & Shenoy, S. K. (2007). β-Arrestins and Cell Signaling. *Annual Review of Physiology*, *69*(1), 483–510. doi:10.1146/annurev.physiol.69.022405.154749

Dewire, S. M., Yamashita, D. S., Rominger, D. H., Liu, G., Cowan, C. L., Graczyk, T. M., … Violin, J. D. (2013). A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared to morphine. *The Journal of pharmacology and experimental therapeutics*. doi:10.1124/jpet.112.201616

Dittman, A. H., Weber, J. P., Hinds, T. R., Choi, E. J., Migeon, J. C., Nathanson, N. M., & Storm, D. R. (1994). A Novel Mechanism for Coupling of m4 Muscarinic Acetylcholine Receptors to Calmodulin-Sensitive Adenylyl Cyclases: Crossover from G Protein-Coupled Inhibition to Stimulation. *Biochemistry*, *33*(4), 943–951. doi:10.1021/bi00170a013

Doi, T., Sugimoto, H., Arimoto, I., Hiroaki, Y., & Fujiyoshi, Y. (1999). Interactions of endothelin receptor subtypes A and B with Gi, Go, and Gq in reconstituted phospholipid vesicles. *Biochemistry*, *38*(10), 3090–9. doi:10.1021/bi981919m

Dupré, D. J., Thompson, C., Chen, Z., Rollin, S., Larrivée, J.-F., Le Gouill, C., … Stanková, J. (2007). Inverse agonist-induced signaling and down-regulation of the platelet-activating factor receptor. *Cellular signalling*, *19*(10), 2068–79. doi:10.1016/j.cellsig.2007.05.015

Dürr, S., Lyer, S., Mann, J., Janko, C., Tietze, R., Schreiber, E., … Alexiou, C. (2012). Real-time cell analysis of human cancer cell lines after chemotherapy with functionalized magnetic nanoparticles. *Anticancer research*, *32*(5), 1983–9.

Eason, M. G., Jacinto, M. T., & Liggett, S. B. (1994). Contribution of ligand structure to activation of alpha 2-adrenergic receptor subtype coupling to Gs. *Molecular pharmacology*, *45*(4), 696–702.

Eason, M. G., Kurose, H., Holt, B. D., Raymond, J. R., & Liggett, S. B. (1992). Simultaneous coupling of alpha 2-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of alpha 2C10, alpha 2C4, and alpha 2C2 adrenergic receptors to Gi and Gs. *The Journal of biological chemistry*, *267*(22), 15795–801.

Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., … Inagami, T. (1998). Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *The Journal of biological chemistry*, *273*(15), 8890–6.

Ehlert, F. J. (2005). Analysis of allosterism in functional assays. *The Journal of pharmacology and experimental therapeutics*, *315*(2), 740–54. doi:10.1124/jpet.105.090886

Eichmann, T., Lorenz, K., Hoffmann, M., Brockmann, J., Krasel, C., Lohse, M. J., & Quitterer, U. (2003). The amino-terminal domain of G-protein-coupled receptor kinase 2 is a regulatory Gbeta gamma binding site. *The Journal of biological chemistry*, *278*(10), 8052–7. doi:10.1074/jbc.M204795200

Fang, Y., Li, G., & Ferrie, A. M. (2007). Non-invasive optical biosensor for assaying endogenous G protein-coupled receptors in adherent cells. *Journal of pharmacological and toxicological methods*, *55*(3), 314–322. doi:10.1016/j.vascn.2006.11.001

Ferguson, S. S., Downey, W. E., Colapietro, A. M., Barak, L. S., Ménard, L., & Caron, M. G. (1996). Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization. *Science (New York, N.Y.)*, *271*(5247), 363–6.

Ferrandon, S., Feinstein, T. N., Castro, M., Wang, B., Bouley, R., Potts, J. T., … Vilardaga, J.-P. (2009). Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nature chemical biology*, *5*(10), 734–42. doi:10.1038/nchembio.206

Fosbrink, M., Aye-Han, N.-N., Cheong, R., Levchenko, A., & Zhang, J. (2010). Visualization of JNK activity dynamics with a genetically encoded fluorescent biosensor. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(12), 5459–64. doi:10.1073/pnas.0909671107

Fraser, C. M., Arakawa, S., McCombie, W. R., & Venter, J. C. (1989). Cloning, sequence analysis, and permanent expression of a human alpha 2-adrenergic receptor in Chinese hamster ovary cells. Evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. *The Journal of biological chemistry*, *264*(20), 11754–61.

Gaddum, J. (1937). The quantitative effects of antagonistic drugs. *Journal of Physiology*, *89*, 7P–9P.

GADDUM, J. H., HAMEED, K. A., HATHWAY, D. E., & STEPHENS, F. F. (1955). Quantitative studies of antagonists for 5-hydroxytryptamine. *Quarterly journal of experimental physiology and cognate medical sciences*, *40*(1), 49–74.

Galandrin, S, & Bouvier, M. (2006). Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol*, *70*(5), 1575–1584. doi:mol.106.026716 [pii] 10.1124/mol.106.026716

Galandrin, Ségolène, Oligny-Longpré, G., Bonin, H., Ogawa, K., Galés, C., & Bouvier, M. (2008). Conformational rearrangements and signaling cascades involved in ligand-

biased mitogen-activated protein kinase signaling through the beta1-adrenergic receptor. *Molecular pharmacology*, *74*(1), 162–72. doi:10.1124/mol.107.043893

Galandrin, Ségolène, Oligny-Longpré, G., & Bouvier, M. (2007). The evasive nature of drug efficacy: implications for drug discovery. *Trends in pharmacological sciences*, *28*(8), 423–30. doi:10.1016/j.tips.2007.06.005

Galés, C., Rebois, R. V., Hogue, M., Trieu, P., Breit, A., Hébert, T. E., & Bouvier, M. (2005). Real-time monitoring of receptor and G-protein interactions in living cells. *Nature methods*, *2*(3), 177–84. doi:10.1038/nmeth743

Gao, H., Sun, Y., Wu, Y., Luan, B., Wang, Y., Qu, B., & Pei, G. (2004). Identification of beta-arrestin2 as a G protein-coupled receptor-stimulated regulator of NF-kappaB pathways. *Molecular cell*, *14*(3), 303–17.

Ge, L., Ly, Y., Hollenberg, M., & DeFea, K. (2003). A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. *The Journal of biological chemistry*, *278*(36), 34418–26. doi:10.1074/jbc.M300573200

George, S. R., Fan, T., Xie, Z., Tse, R., Tam, V., Varghese, G., & O'Dowd, B. F. (2000). Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *The Journal of biological chemistry*, *275*(34), 26128–35. doi:10.1074/jbc.M000345200

Gerits, N., Kostenko, S., Shiryaev, A., Johannessen, M., & Moens, U. (2008). Relations between the mitogen-activated protein kinase and the cAMP-dependent protein kinase pathways: Comradeship and hostility. *Cellular Signalling*, *20*(9), 1592–1607.

Gesty-Palmer, D., Chen, M., Reiter, E., Ahn, S., Nelson, C. D., Wang, S., … Lefkowitz, R. J. (2006). Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *The Journal of biological chemistry*, *281*(16), 10856–64. doi:10.1074/jbc.M513380200

Gether, U., Lin, S., & Kobilka, B. K. (1995). Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes. *The Journal of biological chemistry*, *270*(47), 28268–75.

Ghanouni, P., Gryczynski, Z., Steenhuis, J. J., Lee, T. W., Farrens, D. L., Lakowicz, J. R., & Kobilka, B. K. (2001a). Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *The Journal of biological chemistry*, *276*(27), 24433–6. doi:10.1074/jbc.C100162200

Ghanouni, P., Gryczynski, Z., Steenhuis, J. J., Lee, T. W., Farrens, D. L., Lakowicz, J. R., & Kobilka, B. K. (2001b). Functionally different agonists induce distinct conformations in

the G protein coupling domain of the beta 2 adrenergic receptor. *The Journal of biological chemistry*, *276*(27), 24433–6. doi:10.1074/jbc.C100162200

Goel, R., Phillips-Mason, P. J., Raben, D. M., & Baldassare, J. J. (2002). alpha-Thrombin induces rapid and sustained Akt phosphorylation by beta-arrestin1-dependent and -independent mechanisms, and only the sustained Akt phosphorylation is essential for G1 phase progression. *The Journal of biological chemistry*, *277*(21), 18640–8. doi:10.1074/jbc.M108995200

González-Maeso, J., Yuen, T., Ebersole, B. J., Wurmbach, E., Lira, A., Zhou, M., … Sealfon, S. C. (2003). Transcriptome fingerprints distinguish hallucinogenic and nonhallucinogenic 5-hydroxytryptamine 2A receptor agonist effects in mouse somatosensory cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *23*(26), 8836–43.

Goodman, O. B., Krupnick, J. G., Santini, F., Gurevich, V. V, Penn, R. B., Gagnon, A. W., … Benovic, J. L. (1996). Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature*, *383*(6599), 447–50. doi:10.1038/383447a0

Gorokhovatsky, A. Y., Marchenkov, V. V, Rudenko, N. V, Ivashina, T. V, Ksenzenko, V. N., Burkhardt, N., … Alakhov, Y. B. (2004). Fusion of Aequorea victoria GFP and aequorin provides their Ca(2+)-induced interaction that results in red shift of GFP absorption and efficient bioluminescence energy transfer. *Biochemical and biophysical research communications*, *320*(3), 703–11. doi:10.1016/j.bbrc.2004.06.014

Grammatopoulos, D. K., Randeva, H. S., Levine, M. A., Kanellopoulou, K. A., & Hillhouse, E. W. (2001). Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. *Journal of neurochemistry*, *76*(2), 509–19.

Granier, S., Kim, S., Shafer, A. M., Ratnala, V. R. P., Fung, J. J., Zare, R. N., & Kobilka, B. (2007). Structure and conformational changes in the C-terminal domain of the beta2-adrenoceptor: insights from fluorescence resonance energy transfer studies. *The Journal of biological chemistry*, *282*(18), 13895–905. doi:10.1074/jbc.M611904200

Grecco, H. E., Schmick, M., & Bastiaens, P. I. H. (2011). Signaling from the Living Plasma Membrane. *Cell*, *144*(6), 897–909.

Gregory, K. J., Hall, N. E., Tobin, A. B., Sexton, P. M., & Christopoulos, A. (2010). Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. *The Journal of biological chemistry*, *285*(10), 7459–74. doi:10.1074/jbc.M109.094011

Grisshammer, R., & Hermans, E. (2001). Functional coupling with Galpha(q) and Galpha(i1) protein subunits promotes high-affinity agonist binding to the neurotensin receptor NTS-1 expressed in Escherichia coli. *FEBS letters*, *493*(2-3), 101–5.

Groer, C. E., Tidgewell, K., Moyer, R. A., Harding, W. W., Rothman, R. B., Prisinzano, T. E., & Bohn, L. M. (2007). An opioid agonist that does not induce mu-opioid receptor--arrestin interactions or receptor internalization. *Molecular pharmacology*, *71*(2), 549–57. doi:10.1124/mol.106.028258

Haberstock-Debic, H., Kim, K.-A., Yu, Y. J., & von Zastrow, M. (2005). Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *25*(34), 7847–57. doi:10.1523/JNEUROSCI.5045-04.2005

Hague, C., Bernstein, L. S., Ramineni, S., Chen, Z., Minneman, K. P., & Hepler, J. R. (2005). Selective inhibition of alpha1A-adrenergic receptor signaling by RGS2 association with the receptor third intracellular loop. *The Journal of biological chemistry*, *280*(29), 27289–95. doi:10.1074/jbc.M502365200

Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., & Lefkowitz, R. J. (1998). A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(15), 8496–501.

Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., … Lefkowitz, R. J. (1998). The beta2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange. *Nature*, *392*(6676), 626–30. doi:10.1038/33458

Halls, M. L., & Cooper, D. M. F. (2011). Regulation by Ca2+-signaling pathways of adenylyl cyclases. *Cold Spring Harbor perspectives in biology*, *3*(1), a004143. doi:10.1101/cshperspect.a004143

Harvey, C. D., Ehrhardt, A. G., Cellurale, C., Zhong, H., Yasuda, R., Davis, R. J., & Svoboda, K. (2008). A genetically encoded fluorescent sensor of ERK activity. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(49), 19264–9. doi:10.1073/pnas.0804598105

Hausdorff, W. P., Caron, M. G., & Lefkowitz, R. J. (1990). Turning off the signal: desensitization of beta-adrenergic receptor function. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *4*(11), 2881–9.

Hay, D. L., Poyner, D. R., & Sexton, P. M. (2006). GPCR modulation by RAMPs. *Pharmacology & therapeutics*, *109*(1-2), 173–97. doi:10.1016/j.pharmthera.2005.06.015

He, J., Bellini, M., Inuzuka, H., Xu, J., Xiong, Y., Yang, X., … Hall, R. A. (2006). Proteomic analysis of beta1-adrenergic receptor interactions with PDZ scaffold proteins. *The Journal of biological chemistry*, *281*(5), 2820–7. doi:10.1074/jbc.M509503200

Heitzler, D., Durand, G., Gallay, N., Rizk, A., Ahn, S., Kim, J., … Reiter, E. (2012). Competing G protein-coupled receptor kinases balance G protein and β-arrestin signaling. *Molecular systems biology*, *8*, 590. doi:10.1038/msb.2012.22

Hepler, J. R., Berman, D. M., Gilman, A. G., & Kozasa, T. (1997). RGS4 and GAIP are GTPase-activating proteins for Gq alpha and block activation of phospholipase C beta by gamma-thio-GTP-Gq alpha. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(2), 428–32.

Hermans, E. (2003). Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacology & Therapeutics*, *99*(1), 25–44. doi:10.1016/S0163-7258(03)00051-2

Hermans, E., Saunders, R., Selkirk, J. V, Mistry, R., Nahorski, S. R., & Challiss, R. A. (2000). Complex involvement of pertussis toxin-sensitive G proteins in the regulation of type 1alpha metabotropic glutamate receptor signaling in baby hamster kidney cells. *Molecular pharmacology*, *58*(2), 352–60.

Herrlich, A., Kühn, B., Grosse, R., Schmid, A., Schultz, G., & Gudermann, T. (1996). Involvement of Gs and Gi proteins in dual coupling of the luteinizing hormone receptor to adenylyl cyclase and phospholipase C. *The Journal of biological chemistry*, *271*(28), 16764–72.

Hill, S. J. (2006). G-protein-coupled receptors: past, present and future. *British journal of pharmacology*, *147 Suppl* , S27–37. doi:10.1038/sj.bjp.0706455

Hodgson, L., Shen, F., & Hahn, K. (2010). Biosensors for characterizing the dynamics of rho family GTPases in living cells. *Current protocols in cell biology*, *46*, 14.11.1–14.11–26. doi:10.1002/0471143030.cb1411s46

Hoeppner, C. Z., Cheng, N., & Ye, R. D. (2012). Identification of a nuclear localization sequence in β-arrestin-1 and its functional implications. *The Journal of biological chemistry*, *287*(12), 8932–43. doi:10.1074/jbc.M111.294058

Hoffmann, C., Gaietta, G., Bünemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., … Lohse, M. J. (2005). A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nature methods*, *2*(3), 171–6. doi:10.1038/nmeth742

Houndolo, T., Boulay, P.-L., & Claing, A. (2005). G protein-coupled receptor endocytosis in ADP-ribosylation factor 6-depleted cells. *The Journal of biological chemistry*, *280*(7), 5598–604. doi:10.1074/jbc.M411456200

Hu, L. A., Tang, Y., Miller, W. E., Cong, M., Lau, A. G., Lefkowitz, R. J., & Hall, R. A. (2000). beta 1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of beta 1-adrenergic receptor interaction with N-methyl-D-aspartate receptors. *The Journal of biological chemistry*, *275*(49), 38659–66. doi:10.1074/jbc.M005938200

Huang, J., Chen, S., Zhang, J. J., & Huang, X.-Y. (2013). Crystal structure of oligomeric β(1)-adrenergic G protein-coupled receptors in ligand-free basal state. *Nature structural & molecular biology*. doi:10.1038/nsmb.2504

Huang, P., Steplock, D., Weinman, E. J., Hall, R. A., Ding, Z., Li, J., … Liu-Chen, L.-Y. (2004). kappa Opioid receptor interacts with Na(+)/H(+)-exchanger regulatory factor-1/Ezrin-radixin-moesin-binding phosphoprotein-50 (NHERF-1/EBP50) to stimulate Na(+)/H(+) exchange independent of G(i)/G(o) proteins. *The Journal of biological chemistry*, *279*(24), 25002–9. doi:10.1074/jbc.M313366200

Huwiler, K. G., Machleidt, T., Chase, L., Hanson, B., & Robers, M. B. (2009). Characterization of serotonin 5-hydroxytryptamine-1A receptor activation using a phospho-extracellular-signal regulated kinase 2 sensor. *Analytical biochemistry*, *393*(1), 95–104. doi:10.1016/j.ab.2009.06.018

Imming, P., Sinning, C., & Meyer, A. (2006). Drugs, their targets and the nature and number of drug targets. *Nature reviews. Drug discovery*, *5*(10), 821–34. doi:10.1038/nrd2132

Irannejad, R., Tomshine, J. C., Tomshine, J. R., Chevalier, M., Mahoney, J. P., Steyaert, J., … von Zastrow, M. (2013). Conformational biosensors reveal GPCR signalling from endosomes. *Nature*, *495*(7442), 534–8. doi:10.1038/nature12000

Jaakola, V. P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y. T., Lane, J. R., … Stevens, R. C. (2008). The 2.6 Angstrom Crystal Structure of a Human A2A Adenosine Receptor Bound to an Antagonist. *Science*, *322*(5905), 1211.

Jakubík, J., Janíčková, H., Randáková, A., El-Fakahany, E. E., & Doležal, V. (2011). Subtype differences in pre-coupling of muscarinic acetylcholine receptors. *PloS one*, *6*(11), e27732. doi:10.1371/journal.pone.0027732

Jarpe, M. B., Knall, C., Mitchell, F. M., Buhl, A. M., Duzic, E., & Johnson, G. L. (1998). [D-Arg1,D-Phe5,D-Trp7,9,Leu11]Substance P acts as a biased agonist toward neuropeptide and chemokine receptors. *The Journal of biological chemistry*, *273*(5), 3097–104.

Jarvis, G. E., Bihan, D., Hamaia, S., Pugh, N., Ghevaert, C. J. G., Pearce, A. C., … Farndale, R. W. (2012). A role for adhesion and degranulation-promoting adapter protein in collagen-induced platelet activation mediated via integrin α(2) β(1). *Journal of thrombosis and haemostasis : JTH*, *10*(2), 268–77. doi:10.1111/j.1538-7836.2011.04567.x

Jiang, L. I., Collins, J., Davis, R., Lin, K.-M., DeCamp, D., Roach, T., … Sternweis, P. C. (2007). Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *The Journal of biological chemistry*, *282*(14), 10576–84. doi:10.1074/jbc.M609695200

Jiménez-Sainz, M. C., Murga, C., Kavelaars, A., Jurado-Pueyo, M., Krakstad, B. F., Heijnen, C. J., … Aragay, A. M. (2006). G protein-coupled receptor kinase 2 negatively regulates chemokine signaling at a level downstream from G protein subunits. *Molecular biology of the cell*, *17*(1), 25–31. doi:10.1091/mbc.E05-05-0399

Jin, L. Q., Wang, H. Y., & Friedman, E. (2001). Stimulated D(1) dopamine receptors couple to multiple Galpha proteins in different brain regions. *Journal of neurochemistry*, *78*(5), 981–90.

Johnson, E. A., Oldfield, S., Braksator, E., Gonzalez-Cuello, A., Couch, D., Hall, K. J., … Henderson, G. (2006). Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Molecular pharmacology*, *70*(2), 676–85. doi:10.1124/mol.106.022376

Jones, S. B., Halenda, S. P., & Bylund, D. B. (1991). Alpha 2-adrenergic receptor stimulation of phospholipase A2 and of adenylate cyclase in transfected Chinese hamster ovary cells is mediated by different mechanisms. *Molecular pharmacology*, *39*(2), 239–45.

Kang, J., Shi, Y., Xiang, B., Qu, B., Su, W., Zhu, M., … Ma, L. (2005). A nuclear function of beta-arrestin1 in GPCR signaling: regulation of histone acetylation and gene transcription. *Cell*, *123*(5), 833–47. doi:10.1016/j.cell.2005.09.011

Katanaev, V. L. (2010). The Wnt/Frizzled GPCR signaling pathway. *Biochemistry. Biokhimiĭa*, *75*(12), 1428–34.

Kauffenstein, G., Laher, I., Matrougui, K., Guérineau, N. C., & Henrion, D. (2012). Emerging role of G protein-coupled receptors in microvascular myogenic tone. *Cardiovascular research*, *95*(2), 223–32. doi:10.1093/cvr/cvs152

Kelly, E., Bailey, C. P., & Henderson, G. (2008). Agonist-selective mechanisms of GPCR desensitization. *British journal of pharmacology*, *153 Suppl* , S379–88. doi:10.1038/sj.bjp.0707604

Kenakin, T. (1995). Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends in pharmacological sciences*, *16*(7), 232–8.

Kenakin, Terry. (2004). Principles: receptor theory in pharmacology. *Trends in pharmacological sciences*, *25*(4), 186–92. doi:10.1016/j.tips.2004.02.012

Kenakin, Terry. (2010). Functional Selectivity and Biased Receptor Signaling. *The Journal of pharmacology and experimental therapeutics*. doi:10.1124/jpet.110.173948

Kenakin, Terry, & Christopoulos, A. (2012). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nature reviews. Drug discovery*, *advance on*. doi:10.1038/nrd3954

Kent, R. S., De Lean, A., & Lefkowitz, R. J. (1980). A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Molecular pharmacology*, *17*(1), 14–23.

Kilts, J. D., Gerhardt, M. A., Richardson, M. D., Sreeram, G., Mackensen, G. B., Grocott, H. P., … Kwatra, M. M. (2000). Beta(2)-adrenergic and several other G protein-coupled receptors in human atrial membranes activate both G(s) and G(i). *Circulation research*, *87*(8), 705–9.

Kitano, J., Kimura, K., Yamazaki, Y., Soda, T., Shigemoto, R., Nakajima, Y., & Nakanishi, S. (2002). Tamalin, a PDZ domain-containing protein, links a protein complex formation of group 1 metabotropic glutamate receptors and the guanine nucleotide exchange factor cytohesins. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *22*(4), 1280–9.

Kitano, J., Nishida, M., Itsukaichi, Y., Minami, I., Ogawa, M., Hirano, T., … Nakanishi, S. (2003). Direct interaction and functional coupling between metabotropic glutamate receptor subtype 1 and voltage-sensitive Cav2.1 Ca2+ channel. *The Journal of biological chemistry*, *278*(27), 25101–8. doi:10.1074/jbc.M303266200

Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G., & Lefkowitz, R. J. (1992). Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *The Journal of biological chemistry*, *267*(3), 1430–3.

Klein, W. L., Sullivan, J., Skorupa, A., & Aguilar, J. S. (1989). Plasticity of neuronal receptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *3*(10), 2132–40.

Kobilka, B. K., & Deupi, X. (2007). Conformational complexity of G-protein-coupled receptors. *Trends in pharmacological sciences*, *28*(8), 397–406. doi:10.1016/j.tips.2007.06.003

Koch, W., Inglese, J., Stone, W., & Lefkowitz, R. (1993). The binding site for the beta gamma subunits of heterotrimeric G proteins on the beta-adrenergic receptor kinase. *J. Biol. Chem.*, *268*(11), 8256–8260.

Kofahl, B., & Klipp, E. (2004). Modelling the dynamics of the yeast pheromone pathway. *Yeast (Chichester, England)*, *21*(10), 831–50. doi:10.1002/yea.1122

Kohout, T. a, Nicholas, S. L., Perry, S. J., Reinhart, G., Junger, S., & Struthers, R. S. (2004). Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *The Journal of biological chemistry*, *279*(22), 23214–22. doi:10.1074/jbc.M402125200

Krueger, K. M., Daaka, Y., Pitcher, J. A., & Lefkowitz, R. J. (1997). The role of sequestration in G protein-coupled receptor resensitization. Regulation of beta2-adrenergic receptor dephosphorylation by vesicular acidification. *The Journal of biological chemistry*, *272*(1), 5–8.

Kruse, A. C., Hu, J., Pan, A. C., Arlow, D. H., Rosenbaum, D. M., Rosemond, E., … Kobilka, B. K. (2012). Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature*, *482*(7386), 552–6. doi:10.1038/nature10867

Kühn, B., & Gudermann, T. (1999). The luteinizing hormone receptor activates phospholipase C via preferential coupling to Gi2. *Biochemistry*, *38*(38), 12490–8.

Kühn, B., Schmid, A., Harteneck, C., Gudermann, T., & Schultz, G. (1996). G proteins of the Gq family couple the H2 histamine receptor to phospholipase C. *Molecular endocrinology (Baltimore, Md.)*, *10*(12), 1697–707.

Kwan, D. H. T., Yung, L. Y., Ye, R. D., & Wong, Y. H. (2012). Activation of Ras-dependent signaling pathways by G(14) -coupled receptors requires the adaptor protein TPR1. *Journal of cellular biochemistry*, *113*(11), 3486–97. doi:10.1002/jcb.24225

Ladds, G., & Willars, G. B. (2006). Mammalian RGS proteins: Multifunctional regulators of cellular signalling. *Seminars in Cell & Developmental Biology*, *17*(3), 363–376.

Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S. G., Caron, M. G., & Barak, L. S. (1999). The beta2-adrenergic receptor/ arrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proceedings of the National Academy of Sciences*, *96*(7), 3712–3717. doi:10.1073/pnas.96.7.3712

Lappano, R., & Maggiolini, M. (2011). G protein-coupled receptors: novel targets for drug discovery in cancer. *Nature Reviews Drug Discovery*, *10*(1), 47–60. doi:10.1038/nrd3320

Lawler, O. A., Miggin, S. M., & Kinsella, B. T. (2001). Protein kinase A-mediated phosphorylation of serine 357 of the mouse prostacyclin receptor regulates its coupling to G(s)-, to G(i)-, and to G(q)-coupled effector signaling. *The Journal of biological chemistry*, *276*(36), 33596–607. doi:10.1074/jbc.M104434200

Lee, F. J. S., Xue, S., Pei, L., Vukusic, B., Chéry, N., Wang, Y., … Liu, F. (2002). Dual Regulation of NMDA Receptor Functions by Direct Protein-Protein Interactions with the Dopamine D1 Receptor. *Cell*, *111*(2), 219–230. doi:10.1016/S0092-8674(02)00962-5

Leeb-Lundberg, L. M., Cotecchia, S., DeBlasi, A., Caron, M. G., & Lefkowitz, R. J. (1987). Regulation of adrenergic receptor function by phosphorylation. I. Agonist-promoted desensitization and phosphorylation of alpha 1-adrenergic receptors coupled to inositol phospholipid metabolism in DDT1 MF-2 smooth muscle cells. *The Journal of biological chemistry*, *262*(7), 3098–105.

Leff, P., Scaramellini, C., Law, C., & McKechnie, K. (1997). A three-state receptor model of agonist action. *Trends in pharmacological sciences*, *18*(10), 355–62.

Lefkowitz, R. J., Mullikin, D., & Caron, M. G. (1976). Regulation of beta-adrenergic receptors by guanyl-5'-yl imidodiphosphate and other purine nucleotides. *The Journal of biological chemistry*, *251*(15), 4686–92.

Levy, J. V, & Richards, V. (1965). Inotropic and metabolic effects of three beta-adrenergic receptor blocking drugs on isolated rabbit left atria. *The Journal of pharmacology and experimental therapeutics*, *150*(3), 361–9.

Lima-Fernandes, E., Enslen, H., Camand, E., Kotelevets, L., Boularan, C., Achour, L., … Scott, M. G. H. (2011). Distinct functional outputs of PTEN signalling are controlled by dynamic association with β-arrestins. *The EMBO journal*, *30*(13), 2557–68. doi:10.1038/emboj.2011.178

Limbird, L. E. (1981). Activation and attenuation of adenylate cyclase. The role of GTP-binding proteins as macromolecular messengers in receptor--cyclase coupling. *The Biochemical journal*, *195*(1), 1–13.

Liu, A. M. F., Lo, R. K. H., Lee, M. M. K., Wang, Y., Yeung, W. W. S., Ho, M. K. C., … Wong, Y. H. (2010). Galpha16 activates Ras by forming a complex with tetratricopeptide repeat 1 (TPR1) and Son of Sevenless (SOS). *Cellular signalling*, *22*(10), 1448–58. doi:10.1016/j.cellsig.2010.05.013

Liu, F., Wan, Q., Pristupa, Z. B., Yu, X. M., Wang, Y. T., & Niznik, H. B. (2000). Direct protein-protein coupling enables cross-talk between dopamine D5 and gamma-aminobutyric acid A receptors. *Nature*, *403*(6767), 274–80. doi:10.1038/35002014

Liu, J. J., Horst, R., Katritch, V., Stevens, R. C., & Wüthrich, K. (2012). Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. *Science (New York, N.Y.)*, *335*(6072), 1106–10. doi:10.1126/science.1215802

Liu, S., Premont, R. T., Kontos, C. D., Zhu, S., & Rockey, D. C. (2005). A crucial role for GRK2 in regulation of endothelial cell nitric oxide synthase function in portal hypertension. *Nature medicine*, *11*(9), 952–8. doi:10.1038/nm1289

Lohse, M. J., Benovic, J. L., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1990). beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science (New York, N.Y.)*, *248*(4962), 1547–50.

Lorenz, K., Lohse, M. J., & Quitterer, U. (2003). Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. *Nature*, *426*(6966), 574–9. doi:10.1038/nature02158

Lu, F. F., Su, P., Liu, F., & Daskalakis, Z. J. (2012). Activation of GABA(B) receptors inhibits protein kinase B/glycogen synthase kinase 3 signaling. *Molecular brain*, *5*, 41. doi:10.1186/1756-6606-5-41

Luk, T., Jin, W., Zvonok, A., Lu, D., Lin, X.-Z., Chavkin, C., … Mackie, K. (2004). Identification of a potent and highly efficacious, yet slowly desensitizing CB1 cannabinoid receptor agonist. *British journal of pharmacology*, *142*(3), 495–500. doi:10.1038/sj.bjp.0705792

Luo, X., Zeng, W., Xu, X., Popov, S., Davignon, I., Wilkie, T. M., … Muallem, S. (1999). Alternate coupling of receptors to Gs and Gi in pancreatic and submandibular gland cells. *The Journal of biological chemistry*, *274*(25), 17684–90.

Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., … Lefkowitz, R. J. (1999). Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science (New York, N.Y.)*, *283*(5402), 655–61.

Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., & Lefkowitz, R. J. (2001). Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(5), 2449–54. doi:10.1073/pnas.041604898

Lymperopoulos, A., Rengo, G., & Koch, W. J. (2007). Adrenal adrenoceptors in heart failure: fine-tuning cardiac stimulation. *Trends in molecular medicine*, *13*(12), 503–11. doi:10.1016/j.molmed.2007.10.005

Lynch, M. J., Baillie, G. S., Mohamed, A., Li, X., Maisonneuve, C., Klussmann, E., … Houslay, M. D. (2005). RNA silencing identifies PDE4D5 as the functionally relevant cAMP phosphodiesterase interacting with beta arrestin to control the protein kinase A/AKAP79-mediated switching of the beta2-adrenergic receptor to activation of ERK in HEK293B2 cells. *The Journal of biological chemistry*, *280*(39), 33178–89. doi:10.1074/jbc.M414316200

MacKinnon, A. C., Waters, C., Jodrell, D., Haslett, C., & Sethi, T. (2001). Bombesin and substance P analogues differentially regulate G-protein coupling to the bombesin

receptor. Direct evidence for biased agonism. *The Journal of biological chemistry*, *276*(30), 28083–91. doi:10.1074/jbc.M009772200

Maeda, K., Das, D., Nakata, H., & Mitsuya, H. (2012). CCR5 inhibitors: emergence, success, and challenges. *Expert opinion on emerging drugs*, *17*(2), 135–45. doi:10.1517/14728214.2012.673584

Magalhaes, A. C., Dunn, H., & Ferguson, S. S. G. (2012). Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. *British journal of pharmacology*, *165*(6), 1717–36. doi:10.1111/j.1476-5381.2011.01552.x

Maggi, C. A., Patacchini, R., Meini, S., & Giuliani, S. (1993). Evidence for the presence of a septide-sensitive tachykinin receptor in the circular muscle of the guinea-pig ileum. *European journal of pharmacology*, *235*(2-3), 309–11.

Maguire, M. E., Van Arsdale, P. M., & Gilman, A. G. (1976). An agonist-specific effect of guanine nucleotides on binding to the beta adrenergic receptor. *Molecular pharmacology*, *12*(2), 335–9.

Malbon, C. C., Tao, J., & Wang, H. (2004). AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes. *The Biochemical journal*, *379*(Pt 1), 1–9. doi:10.1042/BJ20031648

Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., … Granier, S. (2012). Crystal structure of the μ-opioid receptor bound to a morphinan antagonist. *Nature*, *485*(7398), 321–6. doi:10.1038/nature10954

Mao, L., Yang, L., Arora, A., Choe, E. S., Zhang, G., Liu, Z., … Wang, J. Q. (2005). Role of protein phosphatase 2A in mGluR5-regulated MEK/ERK phosphorylation in neurons. *The Journal of biological chemistry*, *280*(13), 12602–10. doi:10.1074/jbc.M411709200

Marchese, A., Raiborg, C., Santini, F., Keen, J. H., Stenmark, H., & Benovic, J. L. (2003). The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Developmental cell*, *5*(5), 709–22.

Marrero, M. B., Venema, V. J., Ju, H., Eaton, D. C., & Venema, R. C. (1998). Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2. *The American journal of physiology*, *275*(5 Pt 1), C1216–23.

Mary, S., Damian, M., Louet, M., Floquet, N., Fehrentz, J.-A., Marie, J., … Banères, J.-L. (2012). Ligands and signaling proteins govern the conformational landscape explored by a G protein-coupled receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(21), 8304–9. doi:10.1073/pnas.1119881109

Maurice, P., Kamal, M., & Jockers, R. (2011). Asymmetry of GPCR oligomers supports their functional relevance. *Trends in pharmacological sciences*, *32*(9), 514–20. doi:10.1016/j.tips.2011.05.006

McClean, M. N., Mody, A., Broach, J. R., & Ramanathan, S. (2007). Cross-talk and decision making in MAP kinase pathways. *Nature genetics*, *39*(3), 409–14. doi:10.1038/ng1957

McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., … Lefkowitz, R. J. (2000). Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science (New York, N.Y.)*, *290*(5496), 1574–7.

McDonald, P. H., & Lefkowitz, R. J. (2001). Beta-Arrestins: new roles in regulating heptahelical receptors' functions. *Cellular signalling*, *13*(10), 683–9.

Merriam, L. A., Baran, C. N., Girard, B. M., Hardwick, J. C., May, V., & Parsons, R. L. (2013). Pituitary adenylate cyclase 1 receptor internalization and endosomal signaling mediate the pituitary adenylate cyclase activating polypeptide-induced increase in guinea pig cardiac neuron excitability. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *33*(10), 4614–22. doi:10.1523/JNEUROSCI.4999-12.2013

Metallo, C. M., & Vander Heiden, M. G. (2013). Understanding metabolic regulation and its influence on cell physiology. *Molecular cell*, *49*(3), 388–98. doi:10.1016/j.molcel.2013.01.018

Metra, M., Cas, L. D., di Lenarda, A., & Poole-Wilson, P. (2004). Beta-blockers in heart failure: are pharmacological differences clinically important? *Heart failure reviews*, *9*(2), 123–30. doi:10.1023/B:HREV.0000046367.99002.a4

Miller, T. W., Wang, E. A., Gould, S., Stein, E. V, Kaur, S., Lim, L., … Roberts, D. D. (2012). Hydrogen sulfide is an endogenous potentiator of T cell activation. *The Journal of biological chemistry*, *287*(6), 4211–21. doi:10.1074/jbc.M111.307819

Miller, W. E. (2000). beta -Arrestin1 Interacts with the Catalytic Domain of the Tyrosine Kinase c-SRC. ROLE OF beta -ARRESTIN1-DEPENDENT TARGETING OF c-SRC IN RECEPTOR ENDOCYTOSIS. *Journal of Biological Chemistry*, *275*(15), 11312–11319. doi:10.1074/jbc.275.15.11312

Miller, William E, & Lefkowitz, R. J. (2001). Expanding roles for β-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Current Opinion in Cell Biology*, *13*(2), 139–145. doi:10.1016/S0955-0674(00)00190-3

Minakami, R., Kato, A., & Sugiyama, H. (2000). Interaction of Vesl-1L/Homer 1c with syntaxin 13. *Biochemical and biophysical research communications*, *272*(2), 466–71. doi:10.1006/bbrc.2000.2777

Mo, W., Zhang, L., Yang, G., Zhai, J., Hu, Z., Chen, Y., … Hu, G. (2008). Nuclear beta-arrestin1 functions as a scaffold for the dephosphorylation of STAT1 and moderates the antiviral activity of IFN-gamma. *Molecular cell*, *31*(5), 695–707. doi:10.1016/j.molcel.2008.06.017

Monczor, F., Fernandez, N., Legnazzi, B. L., Riveiro, M. E., Baldi, A., Shayo, C., & Davio, C. (2003). Tiotidine, a histamine H2 receptor inverse agonist that binds with high affinity to an inactive G-protein-coupled form of the receptor. Experimental support for the cubic ternary complex model. *Molecular pharmacology*, *64*(2), 512–20. doi:10.1124/mol.64.2.512

Moore, R. H., Millman, E. E., Godines, V., Hanania, N. A., Tran, T. M., Peng, H., … Clark, R. B. (2007). Salmeterol stimulation dissociates beta2-adrenergic receptor phosphorylation and internalization. *American journal of respiratory cell and molecular biology*, *36*(2), 254–61. doi:10.1165/rcmb.2006-0158OC

Naga Prasad, S. V, Laporte, S. A., Chamberlain, D., Caron, M. G., Barak, L., & Rockman, H. A. (2002). Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. *The Journal of cell biology*, *158*(3), 563–75. doi:10.1083/jcb.200202113

Negishi, M., Irie, A., Sugimoto, Y., Namba, T., & Ichikawa, A. (1995). Selective coupling of prostaglandin E receptor EP3D to Gi and Gs through interaction of alpha-carboxylic acid of agonist and arginine residue of seventh transmembrane domain. *The Journal of biological chemistry*, *270*(27), 16122–7.

Nickolls, S. A., Fleck, B., Hoare, S. R. J., & Maki, R. A. (2005). Functional Selectivity of Melanocortin 4 Receptor Peptide and Nonpeptide Agonists : Evidence for Ligand-Specific Conformational States. *Pharmacology*, *313*(3), 1281–1288. doi:10.1124/jpet.105.083337.1

Nishioka, T., Frohman, M. A., Matsuda, M., & Kiyokawa, E. (2010). Heterogeneity of Phosphatidic Acid Levels and Distribution at the Plasma Membrane in Living Cells as Visualized by a Forster Resonance Energy Transfer (FRET) Biosensor. *The Journal of biological chemistry*, *285*(46), 35979–87. doi:10.1074/jbc.M110.153007

Nobles, K. N., Xiao, K., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., … Lefkowitz, R. J. (2011). Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Science signaling*, *4*(185), ra51. doi:10.1126/scisignal.2001707

Noma, T., Lemaire, A., Naga Prasad, S. V, Barki-Harrington, L., Tilley, D. G., Chen, J., … Rockman, H. A. (2007). Beta-arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *The Journal of clinical investigation*, *117*(9), 2445–58. doi:10.1172/JCI31901

Nygaard, R., Zou, Y., Dror, R. O., Mildorf, T. J., Arlow, D. H., Manglik, A., … Kobilka, B. K. (2013). The Dynamic Process of β(2)-Adrenergic Receptor Activation. *Cell*, *152*(3), 532–42. doi:10.1016/j.cell.2013.01.008

O'Neill, J. S., Maywood, E. S., & Hastings, M. H. (2013). Cellular mechanisms of circadian pacemaking: beyond transcriptional loops. *Handbook of experimental pharmacology*, (217), 67–103. doi:10.1007/978-3-642-25950-0_4

Offermanns, S., Iida-Klein, A., Segre, G. V, & Simon, M. I. (1996). G alpha q family members couple parathyroid hormone (PTH)/PTH-related peptide and calcitonin receptors to phospholipase C in COS-7 cells. *Molecular endocrinology (Baltimore, Md.)*, *10*(5), 566–74.

Offermanns, S., Wieland, T., Homann, D., Sandmann, J., Bombien, E., Spicher, K., … Jakobs, K. H. (1994). Transfected muscarinic acetylcholine receptors selectively couple to Gi-type G proteins and Gq/11. *Molecular pharmacology*, *45*(5), 890–8.

Ogino, Y., Tanaka, K., & Shimizu, N. (1996). Direct evidence for two distinct G proteins coupling with thrombin receptors in human neuroblastoma SH-EP cells. *European journal of pharmacology*, *316*(1), 105–9.

Oldenhof, J., Vickery, R., Anafi, M., Oak, J., Ray, A., Schoots, O., … Van Tol, H. H. (1998). SH3 binding domains in the dopamine D4 receptor. *Biochemistry*, *37*(45), 15726–36. doi:10.1021/bi981634+

Oligny-Longpré, G., Corbani, M., Zhou, J., Hogue, M., Guillon, G., & Bouvier, M. (2012). Engagement of β-arrestin by transactivated insulin-like growth factor receptor is needed for V2 vasopressin receptor-stimulated ERK1/2 activation. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(17), E1028–37. doi:10.1073/pnas.1112422109

Overington, J. P., Al-Lazikani, B., & Hopkins, A. L. (2006). How many drug targets are there? *Nature reviews. Drug discovery*, *5*(12), 993–6. doi:10.1038/nrd2199

Palanche, T., Ilien, B., Zoffmann, S., Reck, M. P., Bucher, B., Edelstein, S. J., & Galzi, J. L. (2001). The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. *The Journal of biological chemistry*, *276*(37), 34853–61. doi:10.1074/jbc.M104363200

Palczewski, K. (2012). Chemistry and biology of vision. *The Journal of biological chemistry*, *287*(3), 1612–9. doi:10.1074/jbc.R111.301150

Palmer, R. K. (2007). The pharmacology and signaling of bitter, sweet, and umami taste sensing. *Molecular interventions*, *7*(2), 87–98. doi:10.1124/mi.7.2.9

Palmer, T. M., Gettys, T. W., & Stiles, G. L. (1995). Differential interaction with and regulation of multiple G-proteins by the rat A3 adenosine receptor. *The Journal of biological chemistry*, *270*(28), 16895–902.

Pan, H.-L., Wu, Z.-Z., Zhou, H.-Y., Chen, S.-R., Zhang, H.-M., & Li, D.-P. (2008). Modulation of pain transmission by G-protein-coupled receptors. *Pharmacology & therapeutics*, *117*(1), 141–61. doi:10.1016/j.pharmthera.2007.09.003

Park, S. H., Das, B. B., Casagrande, F., Tian, Y., Nothnagel, H. J., Chu, M., … Opella, S. J. (2012). Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature*, *491*(7426), 779–83. doi:10.1038/nature11580

Penela, P., Ribas, C., & Mayor, F. (2003). Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cellular Signalling*, *15*(11), 973–981. doi:10.1016/S0898-6568(03)00099-8

Perez, D. M. (2005). From plants to man: the GPCR "tree of life". *Molecular pharmacology*, *67*(5), 1383–4. doi:10.1124/mol.105.011890

Peters, M. F., & Scott, C. W. (2009). Evaluating Cellular Impedance Assays for Detection of GPCR Pleiotropic Signaling and Functional Selectivity. *Journal of Biomolecular Screening*, *14*(3), 246–255. doi:10.1177/1087057108330115

Pfister, C., Chabre, M., Plouet, J., Tuyen, V., De Kozak, Y., Faure, J., & Kuhn, H. (1985). Retinal S antigen identified as the 48K protein regulating light-dependent phosphodiesterase in rods. *Science*, *228*(4701), 891–893. doi:10.1126/science.2988124

Pheng, L. H., Dumont, Y., Fournier, A., Chabot, J.-G., Beaudet, A., & Quirion, R. (2003). Agonist- and antagonist-induced sequestration/internalization of neuropeptide Y Y1 receptors in HEK293 cells. *British journal of pharmacology*, *139*(4), 695–704. doi:10.1038/sj.bjp.0705306

Pierce, K. L., Premont, R. T., & Lefkowitz, R. J. (2002). Seven-transmembrane receptors. *Nature reviews. Molecular cell biology*, *3*(9), 639–50. doi:10.1038/nrm908

Pitcher, J. A., Freedman, N. J., & Lefkowitz, R. J. (1998). G protein-coupled receptor kinases. *Annual review of biochemistry*, *67*, 653–92. doi:10.1146/annurev.biochem.67.1.653

Pitcher, J., Lohse, M. J., Codina, J., Caron, M. G., & Lefkowitz, R. J. (1992). Desensitization of the isolated beta 2-adrenergic receptor by beta-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs via distinct molecular mechanisms. *Biochemistry*, *31*(12), 3193–7.

Pommier, B., Da Nascimento, S., Dumont, S., Bellier, B., Million, E., Garbay, C., … Noble, F. (1999). The cholecystokininB receptor is coupled to two effector pathways through

pertussis toxin-sensitive and -insensitive G proteins. *Journal of neurochemistry*, *73*(1), 281–8.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., & Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*, *402*(6764), 884–8. doi:10.1038/47260

Radisavljevic, Z. (2013). AKT as locus of cancer positive feedback loops and extreme robustness. *Journal of cellular physiology*, *228*(3), 522–4. doi:10.1002/jcp.24167

Rahmeh, R., Damian, M., Cottet, M., Orcel, H., Mendre, C., Durroux, T., … Granier, S. (2012). Structural insights into biased G protein-coupled receptor signaling revealed by fluorescence spectroscopy. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(17), 6733–8. doi:10.1073/pnas.1201093109

Rajagopal, S., Ahn, S., Rominger, D. H., Gowen-MacDonald, W., Lam, C. M., Dewire, S. M., … Lefkowitz, R. J. (2011). Quantifying ligand bias at seven-transmembrane receptors. *Molecular pharmacology*, *80*(3), 367–77. doi:10.1124/mol.111.072801

Rasmussen, Søren G F, Choi, H.-J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., … Kobilka, B. K. (2007). Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature*, *450*(7168), 383–7. doi:10.1038/nature06325

Rasmussen, Søren G F, DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., … Kobilka, B. K. (2011). Crystal structure of the β2 adrenergic receptor-Gs protein complex. *Nature*, *477*(7366), 549–55. doi:10.1038/nature10361

Rasmussen, Søren G. F., Choi, H.-J., Fung, J. J., Pardon, E., Casarosa, P., Chae, P. S., … Kobilka, B. K. (2011). Structure of a nanobody-stabilized active state of the β2 adrenoceptor. *Nature*, *469*(7329), 175–180. doi:10.1038/nature09648

Reiner, S., Ambrosio, M., Hoffmann, C., & Lohse, M. J. (2010). Differential signaling of the endogenous agonists at the {beta}2-adrenergic receptor. *The Journal of biological chemistry*, *285*(46), 36188 –36198. doi:10.1074/jbc.M110.175604

Reiter, E., & Lefkowitz, R. J. (n.d.). GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in endocrinology and metabolism: TEM*, *17*(4), 159–65. doi:10.1016/j.tem.2006.03.008

Ren, D., Villeneuve, N. F., Jiang, T., Wu, T., Lau, A., Toppin, H. A., & Zhang, D. D. (2011). Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(4), 1433–8. doi:10.1073/pnas.1014275108

Richman, J. G., Brady, A. E., Wang, Q., Hensel, J. L., Colbran, R. J., & Limbird, L. E. (2001). Agonist-regulated Interaction between alpha2-adrenergic receptors and spinophilin. *The Journal of biological chemistry*, *276*(18), 15003–8. doi:10.1074/jbc.M011679200

Ritter, S. L., & Hall, R. A. (2009). Fine-tuning of GPCR activity by receptor-interacting proteins. *Nature reviews. Molecular cell biology*, *10*(12), 819–30. doi:10.1038/nrm2803

Robb, S., Cheek, T. R., Hannan, F. L., Haii, L. M., Midgley, J. M., & Evans, P. D. (1994). Agonist-specific coupling of a cloned Drosophila octopamine/tyramine receptor to multiple second messenger systems. *EMBO Journal*, *13*(6), 1325–1330.

Roettger, B., Ghanekar, D., Rao, R., Toledo, C., Yingling, J., Pinon, D., & Miller, L. (1997). Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Molecular pharmacology*, *51*(3), 357.

Rojas, A., & Dingledine, R. (2013). Ionotropic glutamate receptors: regulation by G-protein-coupled receptors. *Molecular pharmacology*, *83*(4), 746–52. doi:10.1124/mol.112.083352

Rosenbaum, D. M., Zhang, C., Lyons, J. a., Holl, R., Aragao, D., Arlow, D. H., … Kobilka, B. K. (2011). Structure and function of an irreversible agonist-β2 adrenoceptor complex. *Nature*, *469*(7329), 236–240. doi:10.1038/nature09665

Rozengurt, E. (2007). Mitogenic signaling pathways induced by G protein-coupled receptors. *Journal of cellular physiology*, *213*(3), 589–602. doi:10.1002/jcp.21246

Sagan, S., Chassaing, G., Pradier, L., & Lavielle, S. (1996). Tachykinin peptides affect differently the second messenger pathways after binding to CHO-expressed human NK-1 receptors. *The Journal of pharmacology and experimental therapeutics*, *276*(3), 1039–48.

Sah, V. P., Seasholtz, T. M., Sagi, S. A., & Brown, J. H. (2000). The role of Rho in G protein-coupled receptor signal transduction. *Annual review of pharmacology and toxicology*, *40*, 459–89. doi:10.1146/annurev.pharmtox.40.1.459

Salazar, N. C., Chen, J., & Rockman, H. A. (2007). Cardiac GPCRs: GPCR signaling in healthy and failing hearts. *Biochimica et biophysica acta*, *1768*(4), 1006–18. doi:10.1016/j.bbamem.2007.02.010

Samama, P., Cotecchia, S., Costa, T., & Lefkowitz, R. J. (1993). A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *The Journal of biological chemistry*, *268*(7), 4625–36.

Samama, P., Pei, G., Costa, T., Cotecchia, S., & Lefkowitz, J. (1994). Negative Antagonists Promote an Inactive Conformation of the beta2-Adrenergic Receptor. *Molecular pharmacology*, *45*, 390–394.

Santos-Alvarez, J., & Sánchez-Margalet, V. (1999). G protein G alpha q/11 and G alpha i1,2 are activated by pancreastatin receptors in rat liver: studies with GTP-gamma 35S and azido-GTP-alpha-32P. *Journal of cellular biochemistry*, *73*(4), 469–77.

Schievella, A. R., Regier, M. K., Smith, W. L., & Lin, L. L. (1995). Calcium-mediated translocation of cytosolic phospholipase A2 to the nuclear envelope and endoplasmic reticulum. *The Journal of biological chemistry*, *270*(51), 30749–54.

Schröder, R., Janssen, N., Schmidt, J., Kebig, A., Merten, N., Hennen, S., … Kostenis, E. (2010). Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. *Nature biotechnology*, *28*(9), 943–949. doi:10.1038/nbt.1671

Scott, C. W., & Peters, M. F. (2010). Label-Free Whole-Cell Assays: Expanding the Scope of GPCR Screening. *Drug discovery today*, *15*(17-18), 704–716. doi:10.1016/j.drudis.2010.06.008

Scott, M. G. H., Pierotti, V., Storez, H., Lindberg, E., Thuret, A., Muntaner, O., … Marullo, S. (2006). Cooperative regulation of extracellular signal-regulated kinase activation and cell shape change by filamin A and beta-arrestins. *Molecular and cellular biology*, *26*(9), 3432–45. doi:10.1128/MCB.26.9.3432-3445.2006

Seguin, L. R., Villarreal, R. S., & Ciuffo, G. M. (2012). AT_2receptors recruit c-Src, SHP-1 and FAK upon activation by Ang II in PND15 rat hindbrain. *Neurochemistry international*, *60*(2), 199–207. doi:10.1016/j.neuint.2011.11.008

Selkirk, J. V, Price, G. W., Nahorski, S. R., & Challiss, R. A. (2001). Cell type-specific differences in the coupling of recombinant mGlu1alpha receptors to endogenous G protein sub-populations. *Neuropharmacology*, *40*(5), 645–56.

Serrano-Vega, M. J., Magnani, F., Shibata, Y., & Tate, C. G. (2008). Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(3), 877–82. doi:10.1073/pnas.0711253105

Shenoy, S K, Han, S., Zhao, Y. L., Hara, M. R., Oliver, T., Cao, Y., & Dewhirst, M. W. (2012). β-arrestin1 mediates metastatic growth of breast cancer cells by facilitating HIF-1-dependent VEGF expression. *Oncogene*, *31*(3), 282–92. doi:10.1038/onc.2011.238

Shenoy, S K, McDonald, P. H., Kohout, T. A., & Lefkowitz, R. J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science (New York, N.Y.)*, *294*(5545), 1307–13. doi:10.1126/science.1063866

Shenoy, Sudha K, Drake, M. T., Nelson, C. D., Houtz, D. a, Xiao, K., Madabushi, S., … Lefkowitz, R. J. (2006). beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *The Journal of biological chemistry*, *281*(2), 1261–73. doi:10.1074/jbc.M506576200

Shenoy, Sudha K, & Lefkowitz, R. J. (2003). Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. *The Journal of biological chemistry*, *278*(16), 14498–506. doi:10.1074/jbc.M209626200

Shenoy, Sudha K, Xiao, K., Venkataramanan, V., Snyder, P. M., Freedman, N. J., & Weissman, A. M. (2008). Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the beta2-adrenergic receptor. *The Journal of biological chemistry*, *283*(32), 22166–76. doi:10.1074/jbc.M709668200

Shi, L. C., Wang, H. Y., Horwitz, J., & Friedman, E. (1996). Guanine nucleotide regulatory proteins, Gq and Gi1/2, mediate platelet-activating factor-stimulated phosphoinositide metabolism in immortalized hippocampal cells. *Journal of neurochemistry*, *67*(4), 1478–84.

Shioda, S., Takenoya, F., Yagi, M., Wang, L., Hori, Y., & Kageyama, H. (2008). Neural networks of several novel neuropeptides involved in feeding regulation. *Nutrition (Burbank, Los Angeles County, Calif.)*, *24*(9), 848–53. doi:10.1016/j.nut.2008.06.016

Shukla, A. K., Xiao, K., & Lefkowitz, R. J. (2011). Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends in biochemical sciences*, *36*(9), 457–69. doi:10.1016/j.tibs.2011.06.003

Siderovski, D. P., & Willard, F. S. (2005). The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. *International Journal of Biological Sciences*, *1*, 51–66.

Smith, F. D., Oxford, G. S., & Milgram, S. L. (1999). Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *The Journal of biological chemistry*, *274*(28), 19894–900.

Smith, S. O. (2012). Insights into the activation mechanism of the visual receptor rhodopsin. *Biochemical Society transactions*, *40*(2), 389–93. doi:10.1042/BST20110751

Soergel, D. G., Subach, R. A., Cowan, C. L., Violin, J. D., & Lark, M. W. (2013). First Clinical Experience with TRV027: Pharmacokinetics and Pharmacodynamics in Healthy Volunteers. *Journal of clinical pharmacology*, *53*(9), 892–9. doi:10.1002/jcph.111

Spengler, D., Waeber, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., & Journot, L. (1993). Differential signal transduction by five splice variants of the PACAP receptor. *Nature*, *365*, 170–175.

Stadel, J. M., DeLean, A., & Lefkowitz, R. J. (1980). A high affinity agonist . beta-adrenergic receptor complex is an intermediate for catecholamine stimulation of adenylate cyclase in turkey and frog erythrocyte membranes. *The Journal of biological chemistry*, *255*(4), 1436–41.

Stallaert, W., Christopoulos, A., & Bouvier, M. (2011). Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors. *Expert Opinion on Drug Discovery*, *6*(8), 811–825. doi:10.1517/17460441.2011.586691

Stanislaus, D., Ponder, S., Ji, T. H., & Conn, P. M. (1998). Gonadotropin-releasing hormone receptor couples to multiple G proteins in rat gonadotrophs and in GGH3 cells: evidence from palmitoylation and overexpression of G proteins. *Biology of reproduction*, *59*(3), 579–86.

Stefan, C. J., Overton, M. C., & Blumer, K. J. (1998). Mechanisms governing the activation and trafficking of yeast G protein-coupled receptors. *Molecular biology of the cell*, *9*(4), 885–99.

Stefan, E., Malleshaiah, M. K., Breton, B., Ear, P. H., Bachmann, V., Beyermann, M., … Michnick, S. W. (2011). PKA regulatory subunits mediate synergy among conserved G-protein-coupled receptor cascades. *Nature communications*, *2*, 598. doi:10.1038/ncomms1605

Stephenson, R. P. (1956). A Modification of Receptor Theory. *British journal of pharmacology*, *11*, 379.

Stout, B. D., Clarke, W. P., & Berg, K. (2002). Rapid desensitization of the serotonin(2C) receptor system: effector pathway and agonist dependence. *The Journal of pharmacology and experimental therapeutics*, *302*(3), 957–62.

Strachan, R. T., Allen, J. A., Sheffler, D. J., & Roth, B. L. (2010). p90 Ribosomal S6 kinase 2, a novel GPCR kinase, is required for growth factor-mediated attenuation of GPCR signaling. *Biochemistry*, *49*(12), 2657–71. doi:10.1021/bi901921k

Sun, Y., McGarrigle, D., & Huang, X.-Y. (2007). When a G protein-coupled receptor does not couple to a G protein. *Molecular bioSystems*, *3*(12), 849–54. doi:10.1039/b706343a

Sunahara, R. K., Dessauer, C. W., & Gilman, A. G. (1996). Complexity and diversity of mammalian adenylyl cyclases. *Annual review of pharmacology and toxicology*, *36*, 461–80. doi:10.1146/annurev.pa.36.040196.002333

Swaminath, G., Deupi, X., Lee, T. W., Zhu, W., Thian, F. S., Kobilka, T. S., & Kobilka, B. (2005). Probing the beta2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *The Journal of biological chemistry*, *280*(23), 22165–71. doi:10.1074/jbc.M502352200

Swaminath, G., Xiang, Y., Lee, T. W., Steenhuis, J., Parnot, C., & Kobilka, B. K. (2004). Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states. *The Journal of biological chemistry*, *279*(1), 686–91. doi:10.1074/jbc.M310888200

Takai, Y., Sasaki, T., & Matozaki, T. (2001). Small GTP-binding proteins. *Physiological reviews*, *81*(1), 153–208.

Takasu, H., Gardella, T. J., Luck, M. D., Potts, J. T., & Bringhurst, F. R. (1999). Amino-Terminal Modifications of Human Parathyroid Hormone (PTH) Selectively Alter Phospholipase C Signaling via the Type 1 PTH Receptor: Implications for Design of Signal-Specific PTH Ligands †. *Biochemistry*, *38*(41), 13453–13460. doi:10.1021/bi990437n

Tang, H., Zhao, Z. J., Landon, E. J., & Inagami, T. (2000). Regulation of calcium-sensitive tyrosine kinase Pyk2 by angiotensin II in endothelial cells. Roles of Yes tyrosine kinase and tyrosine phosphatase SHP-2. *The Journal of biological chemistry*, *275*(12), 8389–96.

Tang, Y., Hu, L. A., Miller, W. E., Ringstad, N., Hall, R. A., Pitcher, J. A., … Lefkowitz, R. J. (1999). Identification of the endophilins (SH3p4/p8/p13) as novel binding partners for the beta1-adrenergic receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(22), 12559–64.

Thomas, W. G., Qian, H., Chang, C. S., & Karnik, S. (2000). Agonist-induced phosphorylation of the angiotensin II (AT(1A)) receptor requires generation of a conformation that is distinct from the inositol phosphate-signaling state. *The Journal of biological chemistry*, *275*(4), 2893–900.

Tian, D., Zhang, W., He, J., Liu, Y., Song, Z., Zhou, Z., … Hu, Y. (2012). Novel, real-time cell analysis for measuring viral cytopathogenesis and the efficacy of neutralizing antibodies to the 2009 influenza A (H1N1) virus. *PloS one*, *7*(2), e31965. doi:10.1371/journal.pone.0031965

Tiberi, M., & Caron, M. G. (1994). High agonist-independent activity is a distinguishing feature of the dopamine D1B receptor subtype. *J. Biol. Chem.*, *269*(45), 27925–27931.

Tohgo, A., Pierce, K. L., Choy, E. W., Lefkowitz, R. J., & Luttrell, L. M. (2002). beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *The Journal of biological chemistry*, *277*(11), 9429–36. doi:10.1074/jbc.M106457200

Tomura, H., Itoh, H., Sho, K., Sato, K., Nagao, M., Ui, M., … Okajima, F. (1997). Betagamma subunits of pertussis toxin-sensitive G proteins mediate A1 adenosine receptor agonist-induced activation of phospholipase C in collaboration with thyrotropin. A novel stimulatory mechanism through the cross-talk of two types of receptors. *The Journal of biological chemistry*, *272*(37), 23130–7.

Tran, P. V, Lachke, S. A., & Stottmann, R. W. (n.d.). Toward a systems-level understanding of the Hedgehog signaling pathway: defining the complex, robust, and fragile. *Wiley interdisciplinary reviews. Systems biology and medicine*, *5*(1), 83–100. doi:10.1002/wsbm.1193

Trester-Zedlitz, M., Burlingame, A., Kobilka, B., & von Zastrow, M. (2005). Mass spectrometric analysis of agonist effects on posttranslational modifications of the beta-2 adrenoceptor in mammalian cells. *Biochemistry*, *44*(16), 6133–43. doi:10.1021/bi0475469

Tsunematsu, T., & Yamanaka, A. (2012). The role of orexin/hypocretin in the central nervous system and peripheral tissues. *Vitamins and hormones*, *89*, 19–33. doi:10.1016/B978-0-12-394623-2.00002-0

Tu, J. C., Xiao, B., Naisbitt, S., Yuan, J. P., Petralia, R. S., Brakeman, P., … Worley, P. F. (1999). Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron*, *23*(3), 583–92.

Tu, J. C., Xiao, B., Yuan, J. P., Lanahan, A. A., Leoffert, K., Li, M., … Worley, P. F. (1998). Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron*, *21*(4), 717–26.

Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., … Mailman, R. B. (2007). Functional Selectivity and Classical Concepts of Quantitative Pharmacology. *Pharmacology*, *320*(1), 1–13. doi:10.1124/jpet.106.104463.Receptor

Urizar, E., Yano, H., Kolster, R., Galés, C., Lambert, N., & Javitch, J. A. (2011). CODA-RET reveals functional selectivity as a result of GPCR heteromerization. *Nature chemical biology*, *7*(9), 624–30. doi:10.1038/nchembio.623

Vaidehi, N., & Kenakin, T. (2010). The role of conformational ensembles of seven transmembrane receptors in functional selectivity. *Current opinion in pharmacology*, *10*(6), 775–781. doi:10.1016/j.coph.2010.09.004

Vásquez, C., & Lewis, D. L. (1999). The CB1 cannabinoid receptor can sequester G-proteins, making them unavailable to couple to other receptors. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *19*(21), 9271–80.

Venkatakrishnan, A. J., Deupi, X., Lebon, G., Tate, C. G., Schertler, G. F., & Babu, M. M. (2013). Molecular signatures of G-protein-coupled receptors. *Nature*, *494*(7436), 185–194. doi:10.1038/nature11896

Vilardaga, J.-P., Steinmeyer, R., Harms, G. S., & Lohse, M. J. (2005). Molecular basis of inverse agonism in a G protein-coupled receptor. *Nature chemical biology*, *1*(1), 25–8. doi:10.1038/nchembio705

Violin, J. D., Dewire, S. M., Yamashita, D., Rominger, D. H., Nguyen, L., Schiller, K., … Lark, M. W. (2010). Selectively Engaging beta-Arrestins at the Angiotensin II Type 1 Receptor Reduces Blood Pressure and Increases Cardiac. *Journal of Pharmacology and Experimental Therapeutics*, *335*(3), 572–579. doi:10.1124/jpet.110.173005.

Vogt, S., Grosse, R., Schultz, G., & Offermanns, S. (2003). Receptor-dependent RhoA activation in G12/G13-deficient cells: genetic evidence for an involvement of Gq/G11. *The Journal of biological chemistry*, *278*(31), 28743–9. doi:10.1074/jbc.M304570200

Wachter, J., Neureiter, D., Alinger, B., Pichler, M., Fuereder, J., Oberdanner, C., … Kiesslich, T. (2012). Influence of five potential anticancer drugs on wnt pathway and cell survival in human biliary tract cancer cells. *International journal of biological sciences*, *8*(1), 15–29.

Wacker, D., Wang, C., Katritch, V., Han, G. W., Huang, X.-P., Vardy, E., … Stevens, R. C. (2013). Structural Features for Functional Selectivity at Serotonin Receptors. *Science*. doi:10.1126/science.1232808

Walters, R. W., Shukla, A. K., Kovacs, J. J., Violin, J. D., Dewire, S. M., Lam, C. M., … Lefkowitz, R. J. (2009). Beta-Arrestin1 mediates nicotinic acid–induced flushing, but not its antilipolytic effect, in mice. *The Journal of Clinial Investigation*, *119*(5), 1312–1321. doi:10.1172/JCI36806.1312

Wang, C., Jiang, Y., Ma, J., Wu, H., Wacker, D., Katritch, V., … Xu, H. E. (2013). Structural Basis for Molecular Recognition at Serotonin Receptors. *Science*. doi:10.1126/science.1232807

Wang, D, Sadée, W., & Quillan, J. M. (1999). Calmodulin binding to G protein-coupling domain of opioid receptors. *The Journal of biological chemistry*, *274*(31), 22081–8.

Wang, Danxin, Surratt, C. K., & Sadée, W. (2002). Calmodulin Regulation of Basal and Agonist-Stimulated G Protein Coupling by the μ-Opioid Receptor (OP3) in Morphine-Pretreated Cell. *Journal of Neurochemistry*, *75*(2), 763–771. doi:10.1046/j.1471-4159.2000.0750763.x

Wang, H., Undie, A., & Friedman, E. (1995). Evidence for the coupling of Gq protein to D1-like dopamine sites in rat striatum: possible role in dopamine-mediated inositol phosphate formation. *Mol. Pharmacol.*, *48*(6), 988–994.

Wang, H.-Y., Tao, J., Shumay, E., & Malbon, C. C. (2006). G-Protein-coupled receptor-associated A-kinase anchoring proteins: AKAP79 and AKAP250 (gravin). *European journal of cell biology*, *85*(7), 643–50. doi:10.1016/j.ejcb.2005.12.003

Warne, T., Edwards, P. C., Leslie, A. G. W., & Tate, C. G. (2012). Crystal structures of a stabilized β1-adrenoceptor bound to the biased agonists bucindolol and carvedilol. *Structure (London, England : 1993)*, *20*(5), 841–9. doi:10.1016/j.str.2012.03.014

Warne, T., Moukhametzianov, R., Baker, J. G., Nehmé, R., Edwards, P. C., Leslie, A. G. W., … Tate, C. G. (2011). The structural basis for agonist and partial agonist action on a β1-adrenergic receptor. *Nature*, *469*(7329), 241–244. doi:10.1038/nature09746

Warne, T., Serrano-Vega, M. J., Baker, J. G., Moukhametzianov, R., Edwards, P. C., Henderson, R., … Schertler, G. F. X. (2008). Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature*, *454*(7203), 486–91. doi:10.1038/nature07101

Watson, C., Chen, G., Irving, P., Way, J., Chen, W. J., & Kenakin, T. (2000). The use of stimulus-biased assay systems to detect agonist-specific receptor active states: implications for the trafficking of receptor stimulus by agonists. *Molecular pharmacology*, *58*(6), 1230–8.

Wei, H., Ahn, S., Shenoy, S. K., Karnik, S. S., Hunyady, L., Luttrell, L. M., & Lefkowitz, R. J. (2003). Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(19), 10782–7. doi:10.1073/pnas.1834556100

Weiss, J. M., Morgan, P. H., Lutz, M. W., & Kenakin, T. P. (1996). The cubic ternary complex receptor-occupancy model. III. resurrecting efficacy. *Journal of theoretical biology*, *181*(4), 381–97. doi:10.1006/jtbi.1996.0139

Wenzel-Seifert, K., & Seifert, R. (2000). Molecular Analysis of beta 2-Adrenoceptor Coupling to Gs-, Gi-, and Gq-Proteins. *Mol. Pharmacol.*, *58*(5), 954–966.

Werry, T. D., Gregory, K. J., Sexton, P. M., & Christopoulos, A. (2005). Characterization of serotonin 5-HT2C receptor signaling to extracellular signal-regulated kinases 1 and 2. *Journal of neurochemistry*, *93*(6), 1603–15. doi:10.1111/j.1471-4159.2005.03161.x

Whistler, J.L, & von Zastrow, M. (1998). Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(August), 9914–9919.

Whistler, Jennifer L, Chuang, H., Chu, P., Jan, L. Y., & von Zastrow, M. (1999). Functional Dissociation of μ Opioid Receptor Signaling and Endocytosis. *Neuron*, *23*(4), 737–746.

White, J. F., Noinaj, N., Shibata, Y., Love, J., Kloss, B., Xu, F., … Grisshammer, R. (2012). Structure of the agonist-bound neurotensin receptor. *Nature*, *490*(7421), 508–13. doi:10.1038/nature11558

Williams, L. T., & Lefkowitz, R. J. (1977). Slowly reversible binding of catecholamine to a nucleotide-sensitive state of the beta-adrenergic receptor. *The Journal of biological chemistry*, *252*(20), 7207–13.

Windh, R. T., Lee, M. J., Hla, T., An, S., Barr, A. J., & Manning, D. R. (1999). Differential coupling of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and H218/Edg-5 to the G(i), G(q), and G(12) families of heterotrimeric G proteins. *The Journal of biological chemistry*, *274*(39), 27351–8.

Wise, A., Sheehan, M., Rees, S., Lee, M., & Milligan, G. (1999). Comparative analysis of the efficacy of A1 adenosine receptor activation of Gi/o alpha G proteins following coexpression of receptor and G protein and expression of A1 adenosine receptor-Gi/o alpha fusion proteins. *Biochemistry*, *38*(8), 2272–8. doi:10.1021/bi982054f

Wisler, J. W., DeWire, S. M., Whalen, E. J., Violin, J. D., Drake, M. T., Ahn, S., … Lefkowitz, R. J. (2007). A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(42), 16657–62. doi:10.1073/pnas.0707936104

Witherow, D. S., Garrison, T. R., Miller, W. E., & Lefkowitz, R. J. (2004). beta-Arrestin inhibits NF-kappaB activity by means of its interaction with the NF-kappaB inhibitor IkappaBalpha. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(23), 8603–7. doi:10.1073/pnas.0402851101

Wittau, N., Grosse, R., Kalkbrenner, F., Gohla, A., Schultz, G., & Gudermann, T. (2000). The galanin receptor type 2 initiates multiple signaling pathways in small cell lung cancer cells by coupling to G(q), G(i) and G(12) proteins. *Oncogene*, *19*(37), 4199–209. doi:10.1038/sj.onc.1203777

Wu, B., Chien, E. Y. T., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., … Stevens, R. C. (2010a). Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science*, *1066*. doi:10.1126/science.1194396

Wu, B., Chien, E. Y. T., Mol, C. D., Fenalti, G., Liu, W., Katritch, V., … Stevens, R. C. (2010b). Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science*, *330*(6007), 1066–1071. doi:10.1126/science.1194396

Xia, Z., & Storm, D. R. (2012). Role of signal transduction crosstalk between adenylyl cyclase and MAP kinase in hippocampus-dependent memory. *Learning & memory (Cold Spring Harbor, N.Y.)*, *19*(9), 369–74. doi:10.1101/lm.027128.112

Xiao, K., McClatchy, D. B., Shukla, A. K., Zhao, Y., Chen, M., Shenoy, S. K., … Lefkowitz, R. J. (2007). Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(29), 12011–6. doi:10.1073/pnas.0704849104

Xiao, R. P., Ji, X., & Lakatta, E. G. (1995). Functional coupling of the beta 2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Molecular pharmacology*, *47*(2), 322–9.

Xu, F., Wu, H., Katritch, V., Han, G. W., Jacobson, K. A., Gao, Z.-G., … Stevens, R. C. (2011). Structure of an agonist-bound human A2A adenosine receptor. *Science (New York, N.Y.)*, *332*(6027), 322–7. doi:10.1126/science.1202793

Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., & Hall, R. A. (2001). beta 1-adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. *The Journal of biological chemistry*, *276*(44), 41310–7. doi:10.1074/jbc.M107480200

Xu, Y., Yan, J., Zhou, P., Li, J., Gao, H., Xia, Y., & Wang, Q. (2012). Neurotransmitter receptors and cognitive dysfunction in Alzheimer's disease and Parkinson's disease. *Progress in neurobiology*, *97*(1), 1–13. doi:10.1016/j.pneurobio.2012.02.002

Yang, D., Chertov, O., & Oppenheim, J. J. (2001). The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. *Cellular and molecular life sciences : CMLS*, *58*(7), 978–89.

Yao, X. J., Vélez Ruiz, G., Whorton, M. R., Rasmussen, S. G. F., DeVree, B. T., Deupi, X., … Kobilka, B. (2009). The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(23), 9501–6. doi:10.1073/pnas.0811437106

Yona, S., Lin, H.-H., & Stacey, M. (2010). Immunity and adhesion-GPCRs. *Advances in experimental medicine and biology*, *706*, 121–7.

Yu, N., Atienza, J. M., Bernard, J., Blanc, S., Zhu, J., Wang, X., … Abassi, Y. a. (2006). Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Analytical chemistry*, *78*(1), 35–43. doi:10.1021/ac051695v

Yun, H.-M., Kim, S., Kim, H.-J., Kostenis, E., Kim, J. Il, Seong, J. Y., … Rhim, H. (2007). The novel cellular mechanism of human 5-HT6 receptor through an interaction with Fyn. *The Journal of biological chemistry*, *282*(8), 5496–505. doi:10.1074/jbc.M606215200

Zak, D. E., & Aderem, A. (2009). Systems biology of innate immunity. *Immunological reviews*, *227*(1), 264–82. doi:10.1111/j.1600-065X.2008.00721.x

Zhang, C., Srinivasan, Y., Arlow, D. H., Fung, J. J., Palmer, D., Zheng, Y., … Kobilka, B. K. (2012). High-resolution crystal structure of human protease-activated receptor 1. *Nature*, *492*(7429), 387–92. doi:10.1038/nature11701

Zhang, J. (1998). Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proceedings of the National Academy of Sciences*, *95*(12), 7157–7162. doi:10.1073/pnas.95.12.7157

Zhang, J., Ferguson, S. S., Barak, L. S., Ménard, L., & Caron, M. G. (1996). Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *The Journal of biological chemistry*, *271*(31), 18302–5.

Zhu, W. Z., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K., & Xiao, R. P. (2001). Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(4), 1607–12. doi:10.1073/pnas.98.4.1607

Zhuang, L., Hu, W., Xin, S., Zhao, J., & Pei, G. (2011). Beta-arrestin-1 protein represses adipogenesis and inflammatory responses through its interaction with peroxisome proliferator-activated receptor-gamma (PPARgamma). *The Journal of biological chemistry*, *286*(32), 28403–13. doi:10.1074/jbc.M111.256099

Zimmerman, B., Beautrait, A., Aguila, B., Charles, R., Escher, E., Claing, A., … Laporte, S. A. (2012). Differential β-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs. *Science signaling*, *5*(221), ra33. doi:10.1126/scisignal.2002522

Zoudilova, M., Kumar, P., Ge, L., Wang, P., Bokoch, G. M., & DeFea, K. A. (2007). Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. *The Journal of biological chemistry*, *282*(28), 20634–46. doi:10.1074/jbc.M701391200

ANNEX I: List of other publications

1) **Stallaert W**, Christopoulos A & Bouvier M. Ligand functional selectivity and quantitative pharmacology at G protein-coupled receptors. *Expert Opin Drug Discov.* 2011 Aug;6(8):811-25. doi: 10.1517/17460441.2011.586691. Epub 2011 May 24. (See Annex II, page *xviii*)

   In this review article, we summarize the current state of research regarding ligand functional selectivity at GPCRs, including a historical perspective, examples of different paradigms of functional selectivity, a mechanistic interpretation and a focus on the use of label-free techniques to study functional selectivity. As first author, I wrote the first draft of the manuscript and participated heavily in its subsequent revision.

2) Audet M, Khoury E, **Stallaert W**, Lukasheva V, Legouill C, Beautrait A, Laporte SA & Bouvier M. βarrestins inhibitor discovery by combined virtual screening and cell-based assays. *In review*.

   This article describes the discovery of a novel inhibitor of β-arrestins using *in silico* methods and its subsequent characterization. As a contributing author, I constructed the β-arrestin-RlucII plasmid used to characterize the effect of the inhibitor in cells. I also participated in numerous discussions with the primary author of the paper, MA, about the interpretation of data and experimental approaches.

3) Bouvier M, Quoyer J, Kobayashi H, Plouffe B, Murat B, **Stallaert W** & Ezzy M. Exploring the nature and function of GPCR oligomerization; a technological challenge and a conceptual revolution. *In preparation for Nature Reviews Chemical Biology.*

   In this review article, we discuss the phenomenon of GPCR oligomerization, detailing its structural basis, functional consequences and potential implication for therapeutic targeting. We also discuss in detail the technical approaches and challenges to studying the oligomerization of GPCRs. As a contributing author, I wrote the section detailing the implication of GPCR oligomerization in ligand functional selectivity.

ANNEX II: Review paper

Ligand Functional Selectivity and Quantitative Pharmacology at G Protein-Coupled Receptors

Expert Opin Drug Discov. 2011 Aug;6(8):811-25. doi: 10.1517/17460441.2011.586691. Epub 2011 May 24

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Abstract:

Introduction: In recent years, it has become clear that individual G protein-coupled receptors (GPCRs) can elicit multiple G protein-dependent and -independent cellular responses. This has led to the discovery that certain ligands can differentially modulate these responses, a concept known as functional selectivity.

Areas covered: In this review, we will describe the various manifestations of functional selectivity and its potential implication in drug discovery. First, a historical perspective of the observations and methodologies that led to the evolution of this concept will be presented. We will then describe the proposed molecular mechanisms responsible for the engagement of distinct subsets of signaling repertoire by different ligands . Finally, this review will offer a synthetic view of how functional selectivity could be used in the design of safer and more effective drugs.

Expert opinion: Our better understanding of the recent the various ways by which compounds modulate GPCR activity has led to a parallel expansion of the terminology used to describe these phenomena. In this review, we propose a standardization of this nomenclature as an essential step to both simplify and clarify the language used among researchers to facilitate future collaboration and discovery at these important therapeutic targets. Such clarification of the various aspects of functional selectivity, coupled to the development of tools to effectively monitor it, will undoubtedly bring this emerging concept into the general paradigm of drug discovery at GPCRs.

Keywords: Efficacy, G protein-coupled receptors, Potency, Selectivity, Signaling

1. Introduction

G protein-coupled receptor (GPCR) pharmacology has been a cornerstone in the evolution of pharmacological theory for decades. With at least 900 individual members in humans[1], these important cell surface receptors are involved in nearly every imaginable aspect of physiology. GPCRs are capable of transducing a cellular response to a vast array of extracellular stimuli, including neurotransmitters, hormones, small peptides, odorants, taste, and even light. Furthermore, GPCRs play a role in a wide variety of pathophysiological processes and consequently are the largest family of therapeutic targets[2].

The ability of a given drug to elicit a response through a receptor was classically proposed to be a product of two distinct molecular properties: the propensity of the compound to bind the receptor ("affinity") and the magnitude of the cellular response generated by the ligand-bound receptor ("intrinsic efficacy")[3]. The discovery of these fundamental molecular properties revolutionized pharmacological research since it allowed a quantitative description of a given ligand-receptor pair that was independent of the system used to measure it. For decades, drugs have been classified according to their affinity and efficacy at a given GPCR target, and these properties are used to predict the therapeutic action of a compound. While ligand affinity offers information about the nature of the interaction between the drug and receptor, which can be used to predict the selectivity of a drug for its target, it is the efficacy of a drug that ultimately determines the nature of the cellular response upon ligand treatment.

Modern evaluation of efficacy typically involves the measurement of a single signaling event downstream of receptor activation (e.g. production of second messengers, activation of ion channels etc.). Due to their simplicity and relatively low cost, such surrogate readouts for receptor activation have been used almost universally in drug screening strategies to identify compounds with a desired pharmacology for a given receptor. Drugs that promote the maximal possible response in a given system are classified as full agonists, while those that generate a sub-maximal response, even at concentrations saturating all receptor sites, are termed partial agonists. Antagonists are said to have zero (intrinsic) efficacy since they simply occupy receptors without eliciting a detectable cellular response. The discovery that many GPCRs elicit constitutive signaling activity in the absence of bound ligand led to the characterization of an additional class of compounds with negative efficacy, called inverse agonists, which reduce the signaling output of the receptor below that of the basal state[4,5].

2. The two-state model of GPCR activation

The ability of GPCRs to translate external stimuli into intracellular signaling activity has traditionally been explained by a two-state model of receptor activation[6-10]. According to this lock-and-key model, GPCRs act as bimodal switches; agonist binding stabilizes the active conformation of the receptor, shifting the receptor equilibrium of the system from a predominantly inactive to an active signaling state. This single active receptor conformation can engage and activate cytoplasmic heterotrimeric G proteins, the major effectors of GPCR-mediated signaling, to elicit signaling events ranging from the production of second messengers (e.g. cAMP, Ca^{2+}, inositol phosphates (IP), etc.), modulation of ion channels, activation of kinase cascades and/or regulation of gene transcription[2]. In the context of a two-state model, agonists and inverse agonists are characterized by their preferential affinities for a given state; inverse agonists and agonists preferentially binding to and stabilizing the

inactive and the active receptor states, respectively. Neutral antagonists would not discriminate between the two conformers and thus would block both agonist and inverse agonist activities.

It is now known that GPCRs can often elicit multiple signaling events upon activation. This functional pleiotropy stems from the ability of GPCRs to couple to a variety of cytoplasmic effectors upon activation (Figure 1), which together mediate and regulate the overall cellular response to receptor stimulation. Many receptors have been shown to promiscuously couple to and activate more than one G protein subtype to elicit multiple G protein-dependent signaling events[11]. Although such promiscuity in G protein coupling was first observed in heterologous expression systems and sometimes interpreted as artifacts of over-expression, it is now clear that coupling of a given receptor to more than one G protein occurs in physiological conditions and is the rule rather than the exception[11]. This represents a fundamental change in our views of GPCR signaling that is epitomized by the fact that we traditionally have categorized receptors by their selective coupling to a given G protein, as, for example, G_s-, G_i- or G_q-coupled. In addition, it is now widely recognized that GPCRs can interact with a vast assortment of non-G protein effectors to elicit various G protein-independent signaling events[12,13]. Receptor activation also promotes the recruitment of various kinases as well as the endocytic machinery of the cell, leading to the phosphorylation and internalization of receptor, respectively. Both mechanisms lead to the desensitization of the signaling capabilities of the receptor, which itself has important consequences on the overall cellular physiology following receptor activation. As researchers continue to identify cellular responses triggered by GPCR activation, many began to question whether the pharmacological properties of a ligand would be conserved over all of the signaling responses that can be engaged by a given receptor. According to the two-state model, which posits that the activity of a ligand is determined by its ability to stabilize a single active receptor conformation, the fundamental molecular properties of a drug should be conserved regardless of the signaling event being measured.

A comprehensive exploration of this question in recent years has revealed a significantly more complex picture of GPCR activation and signaling than previously understood. Several studies have explicitly demonstrated that the pharmacological properties of a compound at a given receptor can differ depending on the signaling event being measured, a phenomenon that is referred to as ‘functional selectivity’, ‘ligand-biased signaling’, or ‘agonist-directed trafficking of stimulus’, among several others (see *Expert Opinion* section for additional discussion on terminology). These findings have clearly demonstrated that GPCR activation cannot be reduced to a simple on/off mechanism, as predicted by the two-state model, and instead suggest the existence of multiple active receptor conformations with distinct signaling abilities. These findings challenge conventional views of receptor activation and offer exciting therapeutic strategies for drug design at GPCRs. In the current review, we will provide an overview of this ligand functional selectivity, highlighting the various ways it is manifested, the therapeutic challenges and benefits it offers, as well as several technical advancements well-suited to study and quantify drug activity in this context.

3. The many facets of functional selectivity

3.1 Ligand-biased signaling

The concept of ligand functional selectivity[14] arose primarily through investigations of the mechanism that could lead to differences observed in the efficacy or potency of drugs towards distinct pathways elicited upon activation of a single receptor. Whereas maximal agonist effect is determined by the intrinsic efficacy of a drug and system stimulus-response coupling factors, agonist potency reflects the relationship between the concentration of drug applied and the cellular response elicited, and is thus additionally influenced by drug affinity. If the affinity and intrinsic efficacy of a given agonist are invariant for a specific receptor, then the most common reason for differences in the absolute potency of an agonist between different pathways has traditionally been ascribed to variation in the aforementioned system stimulus-response coupling factors, such as receptor density or relative amplification of each pathway[15]. The first unequivocal evidence of functional selectivity was the observation that ligands can exhibit reversals in the rank order of potency from one pathway to another[16,17]. Indeed, such inversion in the rank order of potency cannot be explained by differences in the amplification of the signaling pathways or the detection systems used since the impact of these factors should affect all ligands equally. It follows that these data could not be easily reconciled with the 'two-state' model classically used to explain agonism, antagonism and inverse agonism, suggesting instead that different ligands can promote distinct receptor active states with preferences towards different signaling pathways.

It has now been shown for a large number of GPCRs that the 'intrinsic' efficacy of a ligand can differ from one pathway to another[18-21]. One of the original observations came from a study of 5-HT_{2C} receptor signaling[22]. In that study, various 5-HT receptor ligands were assessed for their ability to stimulate two independent pathways leading to IP production and arachidonic acid (AA) accumulation. The elegant design of this study allowed both pathways to be monitored in parallel, negating any confounding effects due to assay-specific conditions that could alter the relative signaling abilities of the drugs. Interestingly, the rank order of efficacy of the compounds was dramatically different from one pathway to the other. Upon stimulation of the 5-HT_{2C}, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), bufotenin and lysergic diethylamide acid (LSD) have greater relative efficacy towards AA accumulation than IP release. On the other hand, two other ligands, 3-trifluoromethylphenyl-piperazine (TFMPP) and quipazine demonstrated greater efficacy for IP release compared to AA accumulation. Such pathway-dependent efficacy has also been shown to occur among receptors with multiple endogenous ligands, such as the β_2AR[23], the chemokine receptor CCR7[24] and the melanocortin MC4 receptor[25].

Although many examples of ligand-biased signaling result in relatively modest differences in agonism between individual pathways, some compounds show more extreme pathway biases, characterized by selective agonism towards one pathway and a lack of activity towards another. In many such cases, these ligands show a clear bias between G protein-dependent and G protein-independent signaling. Many of the best characterized of these G protein-independent pathways involve the scaffolding protein β-arrestin. Indeed, in addition to its established role in promoting receptor internalization, β-arrestin has more recently been shown to be an important effector in various G protein-independent signaling events[26,27]. The TRV120027 peptide for the angiotensin receptor AT_{1A}R is one such example of a ligand with a clear bias toward β-arrestin-dependent signaling. Despite being a neutral

antagonist of G protein-dependent signaling at the $AT_{1A}R$, TRV120027 leads to a robust activation of several β-arrestin-mediated events, including the phosphorylation of ERK1/2, c-Src, eNOS, FAK and c-Jun[28]. Furthermore, TRV120027 showed clear functional distinctions *in vivo* from unbiased $AT_{1A}R$ agonists or antagonists, by promoting both a decrease in mean arterial pressure through the antagonism of G protein-dependent signaling and an increase in cardiomyocyte contractility presumably through agonism of β-arrestin-dependent responses[28]. This functional profile suggests potential therapeutic application in patients with acute heart failure, a condition in which decreased cardiac performance is often accompanied by high blood pressure and an increase in the activity of the renin-angiotensin system. An even more extreme example of ligand-biased signaling is the reversal of efficacy exhibited by several of the 'β-blocker' compounds targeting the β-adrenergic receptors (i.e. β_1AR and β_2AR). The first evidence of such a reversal in the efficacy of a single ligand toward two distinct pathways was the observation that propranolol and ICI118,551, despite being inverse agonists for cAMP production, acted as partial agonists for ERK1/2 activation at the human β_2AR[29]. This behavior is not restricted to the β_2AR, as similar reversals in efficacy have been observed for ligands targeting the $5\text{-}HT_{2C}$ receptor[30], the δ-opioid receptor[31] and the closely-related β_1AR[32], among others. Interestingly, although many β-adrenergic ligands show selective affinities and potencies for β_1AR or β_2AR subtypes, ligands can also exhibit differences in pathway selectivity at each receptor. Labetalol, for example, is considered a non-selective β-adrenergic ligand, with similar affinity for β_1AR and β_2AR, and activating cAMP production with similar potency and efficacy at the two receptor subtypes, yet it activates ERK1/2 in a β_2AR-selective manner, with no activity for ERK1/2 at the β_1AR[32]. This finding illustrates a relatively underappreciated element of ligand selectivity; ligands not only display selectivity at the level of affinity for receptors, but can also differ in the signaling pathways engaged upon binding distinct receptor populations.

Harnessing such pathway selectivity represents a promising avenue in drug discovery. In a clinical trial of chronic heart failure patients, carvedilol, a β-blocker with partial ERK1/2 activity[33], led to a significant reduction in mortality compared to metoprolol, a β-blocker with no activity towards the ERK1/2 pathway[34]. Whether the difference in therapeutic activity relates to the distinctive ability of carvedilol to activate ERK1/2 remains to be investigated but this finding illustrates how ligand-biased signaling could impact future drug discovery strategies. Furthermore, in cases where targeting a given GPCR has been difficult due to adverse effects resulting from the modulation of pathways not related to the pathology, a search for ligands with selectivity for the disease-related pathway may rekindle research programs at targets previously determined to be "undruggable".

Although the above examples of biased agonists for the $AT_{1A}R$ and βARs illustrate ligand-biased signaling considering two distinct pathways, one can imagine expanding upon the number of signaling events monitored, thus increasing exponentially the number of possible compound classes for a given receptor (Figure 2). Such potential diversity in signaling profiles for a given GPCR target highlights the pluridimensionality of efficacy and the exciting potential of exploiting ligand-biased signaling to precisely regulate pathological processes.

3.2 Collateral efficacy

In addition to pathway selectivity, ligand functional selectivity also extends to the ability of ligands to selectively engage the various regulatory systems that are initiated upon GPCR activation, leading to events such as receptor phosphorylation, endocytosis and desensitization. Ligand-bound receptor states can recruit various kinases, including G protein-coupled receptor kinases (GRKs), as well as the second messenger-dependent kinases protein kinase A (PKA) and protein kinase C (PKC), which phosphorylate specific residues on the intracellular loops and carboxy terminal tail of the receptor[35]. Whereas receptor phosphorylation by PKA and PKC primarily desensitizes signaling by inhibiting a productive engagement with G proteins, GRK-dependent phosphorylation also promotes the recruitment of β-arrestin proteins that engage the cellular endocytic machinery and induce receptor internalization[36]. Both phosphorylation mechanisms are used to limit the canonical G protein-dependent signaling activity of a receptor upon sustained presentation of ligand to ensure an appropriate cellular response. This desensitization process of receptor activity was proposed to be linear in nature, with the strength of agonist stimulation determining the speed and magnitude of desensitization and internalization. However, dissociation between signaling engagement and desensitization mechanisms has been documented in several studies[37-46]; a phenomenon referred to as collateral efficacy[47]. For example, angiotensin II, the endogenous agonist for the $AT_{1A}R$, stimulates production of inositol (1,4,5)trisphosphate, receptor phosphorylation and internalization, whereas the peptide analog [Sar^1,Ile^4,Ile^8]AngII (SII) stimulates a robust phosphorylation of the receptor, but does not promote G protein-dependent signaling or $AT_{1A}R$ internalization[48]. Agonists can also promote different mechanisms of receptor desensitization through the selective recruitment of distinct subsets of kinases[49]. Johnson *et al.* (2006)[50] demonstrated that μ-opioid receptor agonists for G protein-coupled inwardly rectifying potassium (GIRK) channel activation induce distinct molecular mechanisms leading to the desensitization of the GIRK response. Indeed, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) and fentanyl promote receptor desensitization by a GRK2-dependent mechanism leading to the recruitment of β-arrestins and the subsequent internalization of the receptor. Morphine, on the other hand, promotes PKC-dependent phosphorylation of the receptor, but does not promote GRK-dependent phosphorylation, recruitment of β-arrestins or internalization[50-54]. Further validation of agonist-specific desensitization mechanisms *in vivo* is provided by the observations that morphine tolerance is not affected in GRK3 knockout mice, whereas tolerance to the μ-opioid receptor agonist fentanyl was significantly reduced in these animals[55].

These studies hint at how such ligand functional selectivity can be exploited in drug design strategies to identify drugs with greater therapeutic effect. Morphine is a very effective antinociceptive agent for acute pain. However, prolonged administration of morphine in a chronic pain setting leads to considerable tolerance to the drug and a need to continually escalate the dose, often leading to various undesirable side effects. The fact that signaling engagement can be separated from desensitization processes reveals the possibility of developing compounds that can activate the pertinent antinociceptive signaling pathways without leading to drug tolerance.

In reality, such a therapeutic strategy may prove more complicated than this. For example, although receptor internalization acts as a desensitizing mechanism in the short-term, this process also initiates the process of receptor resensitization and recycling, whereby the receptor undergoes

dephosphorylation in the cytoplasm before being returned to the cell surface, ready again to respond to ligand stimulation[56]. It has been shown that knock-in mice expressing a mutant form of the μ-opioid receptor that desensitizes, internalizes and recycles in response to morphine[57] experience similar antinociceptive effects without developing tolerance or dependence. These data indicate that the design of a compound with a similar signaling profile to morphine, but that is capable of promoting receptor internalization and recycling through GRK and β-arrestin recruitment, might provide greater therapeutic benefits for chronic pain patients[58]. Alternatively, compounds that have signaling profiles similar to morphine but do not induce desensitization by PKC nor β-arrestin may prove to be yet more efficacious.

4. Molecular mechanisms of ligand functional selectivity

The examples presented above clearly indicate that ligand functional selectivity cannot be reconciled with the idea of a single active signaling state of the receptor. It has instead been proposed that GPCRs do not function simply as bimodal switches and instead can assume multiple active receptor states, each with distinct abilities to engage the cellular signaling repertoire[59]. The proportion of receptors in various active signaling states would therefore determine the overall signaling activity of a receptor at any given time. The fraction of the receptor population in any one conformation is determined by the total energy of that state. Lower energy states would be favorable and thus more highly populated. Ligand binding is proposed to modify the preferred energy states of the receptor by stabilizing specific intramolecular interactions at the cost of others, leading to a change in the distribution of conformations[60]. Ligands with different signaling profiles are proposed to stabilize distinct receptor conformations, a process referred to as 'conformational selection'[59]. Using various biophysical and biochemical techniques, several studies have indeed demonstrated that ligands with different signaling profiles can stabilize distinct receptor conformations[61-68]. Such conformational selectivity can lead to differences in receptor coupling to proximal effectors[66,69-73] and result in the selective activation of distinct downstream signaling events[66,74,75]. A study of β_1AR ligands indicated that isoproterenol promotes a receptor conformation distinct from that of propranolol and bucindolol[75]. Despite being full, partial and inverse agonists, respectively, for G protein-dependent cAMP accumulation, all three ligands are agonists for ERK1/2 activation. Interestingly, these ligands activate different signaling cascades that converge on ERK1/2 activation. While isoproterenol is capable of activating both a G protein-dependent and a β-arrestin-dependent pathway, propranolol and bucindolol show selective activity towards the latter. These results indicate that in addition to eliciting differences in signaling outcome, ligands can selectively activate distinct signaling cascades converging on common downstream events through the stabilization of ligand-specific receptor conformations. Interestingly, if the readout measured is downstream of this convergence, such functional selectivity among ligands may not be detected.

Evidence of multiple receptor conformations can be found in studies of rhodopsin, the photoreceptor found in the rod cells of the retina. Rhodopsin passes through several metastable intermediate conformations in the transition from the completely inactive (dark) state to the active metarhodopsin II state. The activation of rhodopsin is peculiar among GPCRs because its spectral properties allow the direct monitoring of conformational transitions. Another particularity of the rhodopsin system is that, instead of a diffusible ligand stimulus, it is activated upon isomerization of a covalently bound ligand, retinal, in response to light. Isomerization of the full inverse agonist 11-*cis*-

retinal to the all-*trans*-retinal agonist temporarily destabilizes the binding pocket and increases the total energy of the system. The relaxation of the structure involves smaller (e.g. rotations of residue side chains) and larger (e.g. helical movements and rotations) structural changes in the receptor that reduce the total energy of the system and propagate conformational changes from the binding pocket to the cytoplasmic interface with bound effectors. Several studies have indicated that the activation pathways of other Family A GPCRs share many features in common with rhodopsin, including a number of key structural changes[64,76].

More detailed structural knowledge of the ligand-specific conformations that drive functional selectivity would be indispensible in the process of drug design and discovery, permitting the development of compounds with higher degrees of desired signaling bias. The few crystal structures of GPCRs obtained thus far provide a fairly static view of receptor structure and mostly describe inactive conformations stabilized by inverse agonists[77-82]. Recently elucidated full and partial agonist-bound structures[83-85] identify key structural distinctions among receptors bound to ligands with different signaling efficacies. These studies represent key advancements in the understanding of GPCR activation and additional structures bound to pathway-selective ligands will undoubtedly provide key steps in understanding some of the important structural determinants in ligand functional selectivity. Yet, crystal structures only provide snapshots of individual ligand-bound structures and cannot probe the dynamic structural changes from one conformation to another. Recent advancements in the use of nuclear magnetic resonance spectroscopy to study GPCRs[86,87] should provide insight into such conformational flexibility in receptor activation and may eventually provide clues about the role of structural dynamics in the ligand functional selectivity.

5. Experimental detection of ligand functional selectivity

Exploiting ligand functional selectivity for therapeutic benefit represents a promising opportunity for the design of safer and more efficacious drugs. Most traditional drug discovery strategies, however, rely on a single signaling readout to assess the activity of compounds at a given target and thus are not adequately suited to detect the full repertoire of possible signaling responses. These screens increase the chances of overlooking compounds that do not activate the specific pathway being monitored but may have meaningful pharmacology at the target. These compounds may prove, in fact, to have a signaling profile of greater therapeutic benefit than those detected in the screen. Several strategies have been developed to probe for ligand functional selectivity in the process of drug discovery, each with its own advantages and disadvantages. Certainly, obtaining a more complete description of ligand activity could be achieved by using multiple screening assays with different, and potentially overlapping, endpoints[47]. Instead of identifying ligands that simply activate or not a given pathway, such a strategy has the advantage of being able to identify compounds with various signaling profiles over multiple signaling events, with the total number of possible compound classes increasing exponentially with the number of readouts being measured[32]. However, aside from the increased cost and time associated with performing multiple screens to assess individual signaling events, differences in assay conditions may introduce confounding factors that obfuscate ligand activity from one assay to another. One approach that has recently been employed, at least with respect to G protein-dependent biased signaling, has made use of different strains of the yeast, *S. cerevisiae,* genetically modified to express different chimeras between

the endogenous yeast Gα protein (Gpa1) and each of the main classes of human Gα subunits[88-90]. This approach, which effectively "hijacks" the yeast's internal pheromone-response growth pathway, can be conveniently linked to a reporter gene output and thus ligand activity can be easily profiled across different yeast strains under similar assay conditions to detect possible biased signaling. Of course, this approach has its own limitations. For instance, not all GPCRs can be easily expressed in yeast, G protein-independent signaling cannot be profiled in a similar manner, and any findings made in this cell type must still be validated in mammalian cells. Nonetheless, this approach has recently led to the discovery that the classic muscarinic receptor antagonist, atropine, is a biased agonist through $G_{12/13}$ signaling[89], and that the allosteric ligand, brucine, can promote pathway-biased allosteric modulation at the M_3 muscarinic receptor[88].

Another means to circumvent some of the problems mentioned above is to evaluate multiple signaling events in a single assay. Biosensors based on fluorescence and bioluminescence resonance energy transfer (FRET and BRET) technology may provide such a solution. These biosensors allow the real-time monitoring of signaling events such as receptor-effector interactions, production of second messengers, activation of ion channels or phosphorylation of signaling proteins in living cells[91-102]. By designing biosensors with unique emission spectra, distinct signaling events can be spectrally separated allowing the multiplexing of various biosensors in a single assay[103]. The continual development of novel biosensors monitoring an ever-increasing number of signaling events as well as technical improvements in FRET and BRET to increase the magnitude and separation signals will certainly make this approach an attractive means to screen for ligand functional selectivity in drug discovery programs.

Regardless of whether screening strategies assess multiple signaling outcomes separately or simultaneously, these methods contain a systematic observational bias; the events being monitored are specifically selected and measured explicitly. This presents a non-trivial technical challenge to obtain an exhaustive measure of ligand activity. To do so, all possible signaling events elicited upon receptor activation must be known and individual assays must be designed, validated and optimized to measure each independently. An alternative strategy to obtain a more holistic description of ligand activity is represented by recently developed technologies that provide an integrative measure of signaling activity by monitoring higher-order changes in cellular activity[104].

One such methodology involves monitoring changes in cellular impedance upon receptor stimulation[105]. By measuring differences in the electrical impedance imposed upon cellular attachment to electrodes in microtiter plates, these platforms are capable of monitoring all signaling events that converge on cellular processes that affect cell morphology, adhesion or cytoskeletal dynamics. Another integrative assay system gaining popularity is based on dynamic mass redistribution (DMR), which uses an optical biosensor to measure spatiotemporal changes in biomass representative of a reorganization of cellular constituents[106]. Both technologies reveal complex response curves that are resolved in real-time in living cells, reflecting an overall cellular response to receptor stimulation. Both impedance- and DMR-based platforms have been shown to effectively detect cellular responses elicited by all four families of G proteins (i.e. G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$). In fact, it has been proposed that qualitative differences in these responses may be predictive of the specific G protein effector system engaged[104]. However the cell-type specificity of these responses[107] combined with the fact that many GPCRs are capable of engaging multiple G protein subtypes challenges such a simple prediction. In fact, impedance

and DMR responses are an integration of multiple signaling events (G protein-dependent and – independent) on the overall cellular response[107,108]. The ability to dissect the contribution of multiple signaling events from an overall response would be an obvious advantage of these techniques to study ligand functional selectivity, in particular if it can be shown that ligands engaging distinct effector systems produce qualitatively different responses. If so, drug screening with these integrative platforms may not only identify hits for a given receptor target, but may identify multiple compound classes with distinct signaling profiles. An additional strength of these integrative assay platforms is that they are amenable to the study of endogenously expressed GPCRs in primary cells[107], allowing ligand activity to be measured in a native receptor context. This also opens the door to studying ligand activity in a pathophysiological context, in which primary cells are isolated from disease models and challenged with compounds. Such studies could offer further insight in the role of ligand functional selectivity in altered signaling systems.

Both cellular impedance and DMR conspicuously resemble classical pharmacological assays where overall ligand effect, encompassing all of the effector systems engaged, was measured by whole-cell or organ response. These newer integrative systems, of course, are scaled down to offer incomparable increases in throughput, thus permitting their use in screening applications[109,110]. However, these newer technologies offer some of the same challenges as classic techniques in that the precise signaling events mediating the cellular responses observed are not explicitly revealed.

6. Quantifying ligand-biased signaling

Given that new examples of ligand-biased signaling are continually being observed, an important challenge to modern drug discovery is the means by which this phenomenon can be readily quantified in a manner that facilitates objective comparisons between ligands across different signal pathways. This is particularly pertinent if a certain profile of biased agonism is deemed to reflect a desirable therapeutic effect, in which case medicinal chemists can work to specifically optimize this profile. Since extreme cases of ligand-biased signaling are characterized by reversals in agonist potency orders or maximal agonist effects between different pathways, it is perhaps not surprising that most studies to date have focused on changes in one or the other of these empirical properties as key quantitative hallmarks of functional selectivity. However, this approach is sub-optimal for at least two reasons. First, not all instances of ligand-biased signaling need be characterized by actual reversals in potency or efficacy orders. The endogenous agonist of a given receptor will have a specific repertoire of coupling preferences that may vary between pathways and cell types. This variability will reflect i) the intrinsic bias of the ligand due to receptor conformational selection and/or ii) cell-dependent differences in signal amplification and/or receptor expression (as outlined previously). The former is the actual determinant of "ligand bias" at the molecular level, whereas the latter will influence both the observed potency (e.g., EC_{50}) and maximal effect (i.e., E_{max}) of a ligand due to purely system-dependent properties. As a consequence of this latter type of "system bias", natural variations in absolute agonist potencies and maximal effects between pathways and assay systems are to be expected. However, if the variation between pathways for a series of ligands does not "track" with the variation of the physiologically relevant (i.e. endogenous) agonist, then this is a sufficient indicator that one or more of the compounds under investigation are exhibiting ligand-biased signaling; reversals in potency/efficacy orders are simply

the extreme manifestation of this situation. Second, in addition to system-dependent influences on ligand behavior, the potency of a ligand towards a given pathway is determined by both its affinity for the receptor state governing that pathway and its "intrinsic" efficacy for generating stimulus to that pathway; maximal agonist effect is only determined by the intrinsic efficacy but not the affinity. Thus, potency and maximal effect emphasize different aspects of a ligand's overall capacity to elicit a response, and unless *both* are incorporated into any analysis of ligand-biased signaling, important information is likely to be lost.

From the preceding discussion, it is apparent that the entire concentration-response relationship of an agonist at a given pathway should be incorporated into any analysis of ligand-biased signaling. A number of recent studies have begun to explore this issue from two related perspectives: how to readily visualize/display ligand-biased signaling, and how to quantify it in a manner that is robust and amenable to statistical evaluation[111-115]. With regards to visualizing ligand-biased signaling, it is clear that the generation of large families of agonist concentration-response data (as is done routinely nowadays in many assay formats) can become rather unwieldy once the data are all plotted together. One means of distilling this information into a more manageable visual display is by converting the data into a "Bias Plot", specifically by plotting the effects to equimolar concentrations of an agonist at one signal pathway relative to the effects at another[111-113]. An example of this approach is illustrated in Figures 3A and 3B, where the effects of four different α_1 adrenergic receptor agonists (norepinephrine, epinephrine, oxymetazoline and phenylephrine) are compared in terms of their ability to modulate whole-cell extracellular acidification rate (E.C.A.R.) versus intracellular calcium mobilization. It should be noted that there is no overt evidence of reversals in potency or efficacy orders; indeed, the Bias Plot **b** indicates that all agonists have a preference towards calcium mobilization relative to E.C.A.R. However it can also be seen from this plot that the agonists do not "track" with one another with regards to this preference. In particular, oxymetazoline and phenylephrine display a clear preference towards E.C.A.R. compared to the two endogenous agonists[115]; this is presumptive evidence that the synthetic agonists may be biased. However, the Bias Plot still retains the influence of system bias, in addition to ligand bias, and thus a more objective means is required to nullify the impact of the former and objectively quantify the latter.

Ideally, analytical approaches to quantifying ligand-biased signaling should be applicable in the most common experimental situations. This means that the technique utilized should not require detailed information of the myriad mechanisms underlying the signal bias and should be applicable to routinely derived concentration-response data. One of the most important analytical tools in pharmacology that satisfies these criteria is the so-called "Operational Model of Agonism", first derived by Black and Leff (1983). This model treats the entire stimulus-response cascade of a receptor-linked signaling pathway as a "virtual enzyme" system, with a Michaelis-Menten-type constant, K_E, that defines the intrinsic efficacy of an activated ligand-receptor complex. Because the observed response will also be influenced by the total number of receptors ($[R]_T$) in the system, the ratio of $[R]_T/K_E$ yields an overall operational efficacy parameter, τ. This operational efficacy parameter, together with the dissociation constant (K_A) of the agonist for the receptor, are sufficient to quantify the overall activity of an agonist for a given cellular pathway; both K_A and τ will contribute to agonist potency, whereas only τ will contribute to the maximal

agonist effect. The extension of this model to the quantification of functional selectivity simply requires the additional assumption that ligand-bias is characterized by *different affinities* and *different intrinsic efficacies*[1] for different active states (i.e. different K_A and different K_E (and, hence, τ) values). This is illustrated schematically in Figure 3C for a theoretical signaling cascade linked to a receptor conformation denoted as R*. Importantly, although the individual values of these parameters are not easily obtainable from simple concentration-response data, the *ratio* of the two, defined as the "transducer ratio" (τ/K_A) in Figure 3C, can almost always be obtained from direct fitting of the operational model to a family of agonist curves at a given pathway[114-116]. Once this is done, all that is necessary to quantify ligand bias is to normalize agonist transducer ratios at each pathway to those of a reference agonist, thus canceling system-dependent influences (as they are common to all the agonists), and then compare these normalized transducer ratios between pathways for each agonist. This is shown in Figure 3D for the four α_1 adrenergic receptor agonists; for statistical purposes[117], the transducer ratios for each pathway are estimated as logarithms ($Log\tau/K_A$), normalized to the value of a reference agonist (norepinephrine in this instance) to yield $\Delta Log(\tau/K_A)$ values at each pathway, and then compared across pathways for each agonist in the form of a LogBias Factor (i.e. $\Delta\Delta Log(\tau/K_A)$). From the example presented in Figure 3D, relative to norepinephrine, oxymetazoline has a statistically significant 8.2-fold bias towards E.C.A.R. and phenylephrine has a 21-fold bias in the same direction. In contrast, the other endogenous agonist, epinephrine, does not display any ligand-bias between the pathways[115]. Although the full potential, and limitations, of such operational approaches to quantify ligand-biased signaling remain to be fully explored, it is clear that the determination of bias factors may provide a robust scale that can facilitate future structure-activity studies focused specifically on exploiting functional selectivity; the information is already encoded in the concentration-response relationship, and it is simply a matter of teasing it out.

7. Conclusion

The functional selectivity of GPCR ligands clearly represents an exciting opportunity for drug discovery programs. The ability to selectively modulate the activity of the pathway responsible for a given pathology without affecting cellular functions unrelated to the disease could lead to the development of safer and more effective drugs (Figure 4). A greater understanding of the molecular mechanisms underlying ligand functional selectivity, through the development of novel approaches to observe and quantify ligand bias, will undoubtedly be of great assistance in informing and expanding such therapeutic strategies.

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[1] The term "intrinsic" efficacy thus becomes a misnomer in this regard.

8. Expert opinion

The pharmacology described in this review represents a paradigm shift in the way that researchers must think about drug activity at GPCRs. However, as the diversity of drug activity continues to be revealed, the terminology used to describe such behavior has also become less clearly defined. Indeed, many terms have been applied to the various manifestations of this phenomenon, often with overlapping, ambiguous or identical meanings. There is a need to clarify the terminology used to describe these recently discovered pharmacological properties. We propose the following vernacular, consisting of both general and specific terms, to describe the various aspects of GPCR pharmacology described in the current review.

First, we propose that functional selectivity be used as a general term to describe all possible instances of ligands differentially influencing receptor behavior, including differences in pathway engagement, receptor post-translational modifications, interactions with effectors, or receptor regulation. Of all the terms previously applied to describe this phenomenon, we feel that functional selectivity is explicitly descriptive of the various ways this phenomenon can be manifested.

More explicit terms should be used to describe specific subsets of such functional selectivity. The term ligand-biased signaling would be used in instances where the pharmacological properties of a ligand-receptor pair differ among the pathways engaged, in a manner that is not simply a function of differences in system stimulus-response coupling factors. This may be reflected by differences in the rank order of efficacies and/or potencies of compounds towards various independent pathways initiated by a single receptor subtype.

The term collateral efficacy can be considered as a special case of ligand-biased signaling as it would refer to differences in receptor regulatory events such as phosphorylation, desensitization and/or receptor endocytosis that influence the strength, duration and/or localization of the signaling response(s). This could be exemplified by two compounds with identical efficacies towards signaling pathways, yet differing in their relative abilities to promote desensitization and/or endocytosis. This term may also apply to differences in the fate of the internalized receptor in response to distinct ligands, leading to degradation or recycling pathways, for example.

Considerable evidence exists to suggest that functional selectivity results from differential stabilization of ligand-bound receptor conformations, as described in this review. We propose that the term conformational selection continue to be used to conceptualize this mechanism. We have also described how these structural distinctions in ligand-receptor pairs can result in the differential engagement of proximal effectors that interact with the receptor. Such effector selection determines the overall cellular response elicited by a given ligand, resulting in ligand-biased signaling and/or collateral efficacy.

Given the complexity described above, it is clear that generic terms such as agonist, inverse agonist or neutral antagonist are no longer sufficient to capture the complete functional repertoire of a given ligand. Yet, it is not practical to invent new terms that attempt to embody the pluridimensionality of ligand activity. Instead, it would be clearer to simply use the existing terms but to qualify them with an explicit cellular event. For example, a compound could be an inverse agonist for signaling pathway A, a neutral antagonist for signaling pathway B and an agonist for receptor endocytosis.

Many of the terms described in this review have been used previously, sometimes in slightly different contexts to what is proposed here. However, we hope that this effort to specify and differentiate among this terminology will help standardize the language used in the field, to remove excess ambiguity and provide a common linguistic platform for researchers in this exciting field.

One of the limiting factors in considering functional selectivity in GPCR drug discovery programs is the difficulty of assessing the full spectrum of receptor activity in a rapid and exhaustive manner. The development of assays that monitor multiple signaling outcomes in homogeneous and multiplexed formats as well as various label-free technologies that provide an integrative assessment of the overall response represent exciting developments that should help overcome this obstacle.

Article Highlights

- GPCRs can assume multiple "active" states that differ in their ability to couple to various G protein and non-G protein effectors and elicit intracellular signaling events

- "Functional selectivity" refers to the ability of GPCR ligands to differentially stabilize distinct active receptor conformations, which may result in differential activity towards certain signaling events at the level of ligand efficacy and/or potency

- Compounds that selectively modulate pathologically-relevant signaling pathways may prove to be highly effective therapies by minimizing adverse effects or the development of tolerance

- Technologies that provide a rapid and holistic assessment of compound activity at a given receptor will be indispensible in exploiting functional selectivity for drug discovery at GPCRs

- A standardization of the nomenclature reflecting these recent conceptual advancements in GPCR pharmacology is essential to reduce ambiguity and increase shared knowledge in the field of drug discovery at GPCRs

Figures

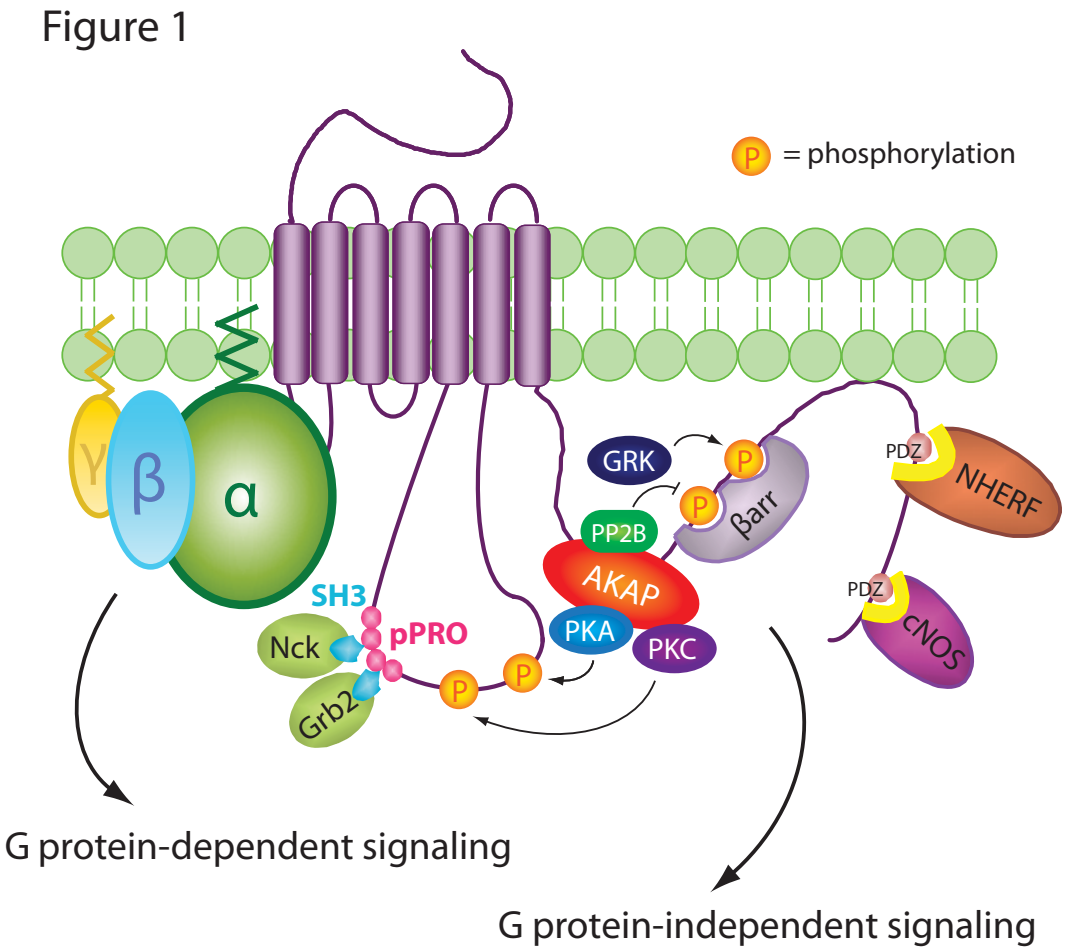

Figure 1 – GPCRs as signaling hubs. Through interactions with numerous cytoplasmic proteins, a wide variety of cellular responses can be elicited upon receptor activation. Ligands can stimulate various G protein-dependent signaling events by promoting the activation of one or more G protein subtypes (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$). Furthermore, additional G protein-independent signaling can be elicited via interactions with non-G protein effectors such as β-arrestin (β-arr). Ligand binding also promotes the recruitment of various kinases (e.g. PKA, PKC, GRKs) and phosphatases (e.g. PP2B) that regulate the phosphorylation state of the receptor. These phosphorylation events often serve to desensitize signaling by decoupling effector interactions and recruiting the cellular endocytic machinery to the receptor; however, receptor phosphorylation can also lead to the recruitment of effectors to initiate additional signaling. Several of these important effector molecules are tethered to a receptor by a shared molecular scaffold, such as the AKAP proteins, which help ensure the spatial and functional coordination of effector responses. *Receptor-effector interactions shown are not meant to represent the actual spatial arrangement of signaling complexes in scale or position.*

Figure 2

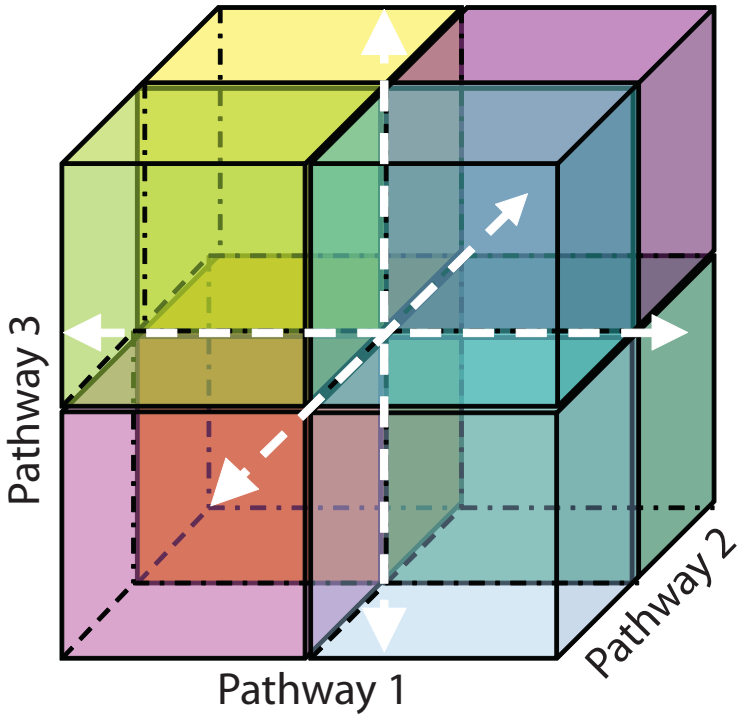

Figure 2 – GPCR signaling pluridimensionality. Since ligand-biased signaling demonstrates that the pharmacology of a ligand can differ among signaling pathways, the categorization of ligands at a given GPCR must incorporate their activity towards all possible signaling events engaged by the receptor. Thus, the number of possible compound classes for a given receptor increases exponentially with the number of signaling readouts measured. If we consider only agonism or inverse agonism towards three independent signaling pathways, as shown here, we obtain eight theoretical compound classes for this receptor. The number of possible efficacy profiles for a given compound is 2^n, *n* being the number of signaling pathways considered. If we consider neutral antagonism or partial responses for each pathway, as is likely the case, even greater ligand signaling texture is possible.

Figure 3

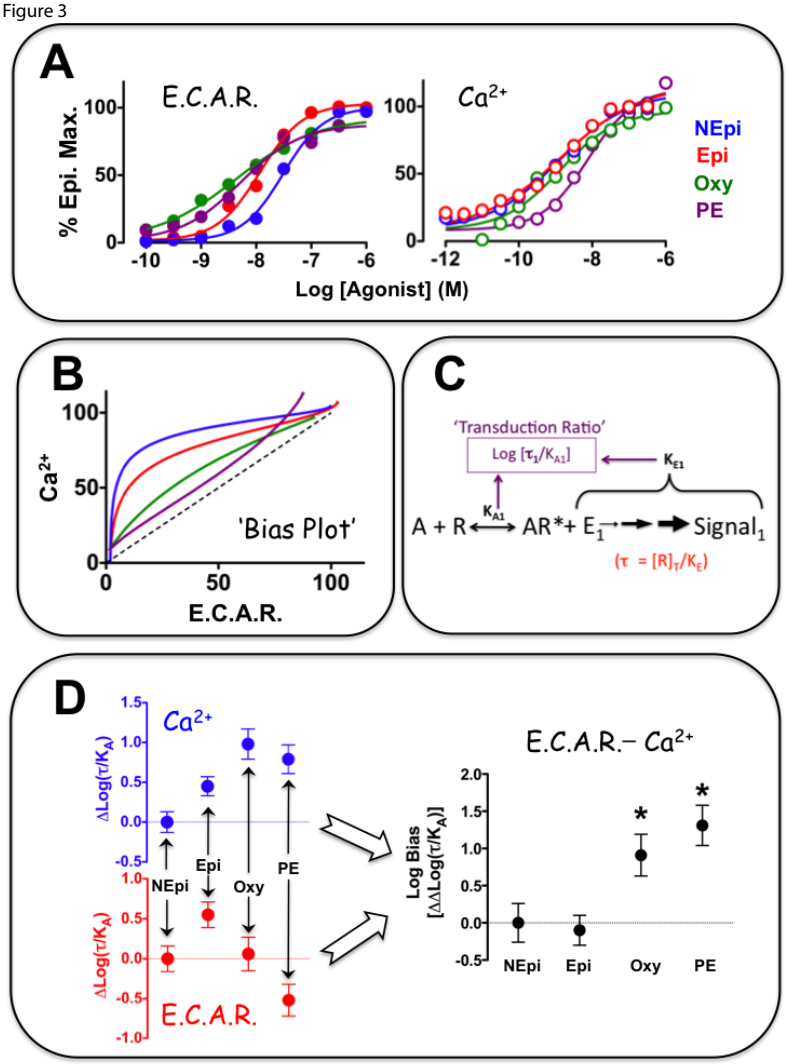

Figure 3 - Display and quantification of ligand-biased signaling. **A.** Concentration-response relationships to norepinephrine (NEpi), epinephrine (Epi), oxymetazoline (Oxy) and phenylephrine (PE) for mediating whole-cell extracellular acidification rate (E.C.A.R; determined by microphysiometry) or intracellular calcium mobilization. Data re-plotted from ref. 114. **B.** Bias plot of the data shown in panel A; note the divergence of oxymetazoline and phenylephrine from the preferences of the two endogenous agonists. **C.** Schematic highlighting the parameters of the operational model of agonism as applied to an individual cellular signaling pathway linked to a receptor conformation denoted as R*. Agonist, A, binds to this state according to its dissociation constant, K_{A1}, promoting coupling to an effector, E_1, which triggers a cascade culminating in the observed $Signal_1$, the efficiency of which is determined by the intrinsic efficacy parameter, K_{E1}. Normalization of the K_E parameter to total receptor density ($[R]_T$) yields the operational efficacy parameter, τ_1. **D.** Determination of normalized $\Delta Log(\tau/K_A)$ values for the indicated agonists at each pathway and use of these values to define the final bias factor ($\Delta\Delta Log(\tau/K_A)$) for each agonist between the two pathways. Asterisks indicate statistical significance.

Figure 4

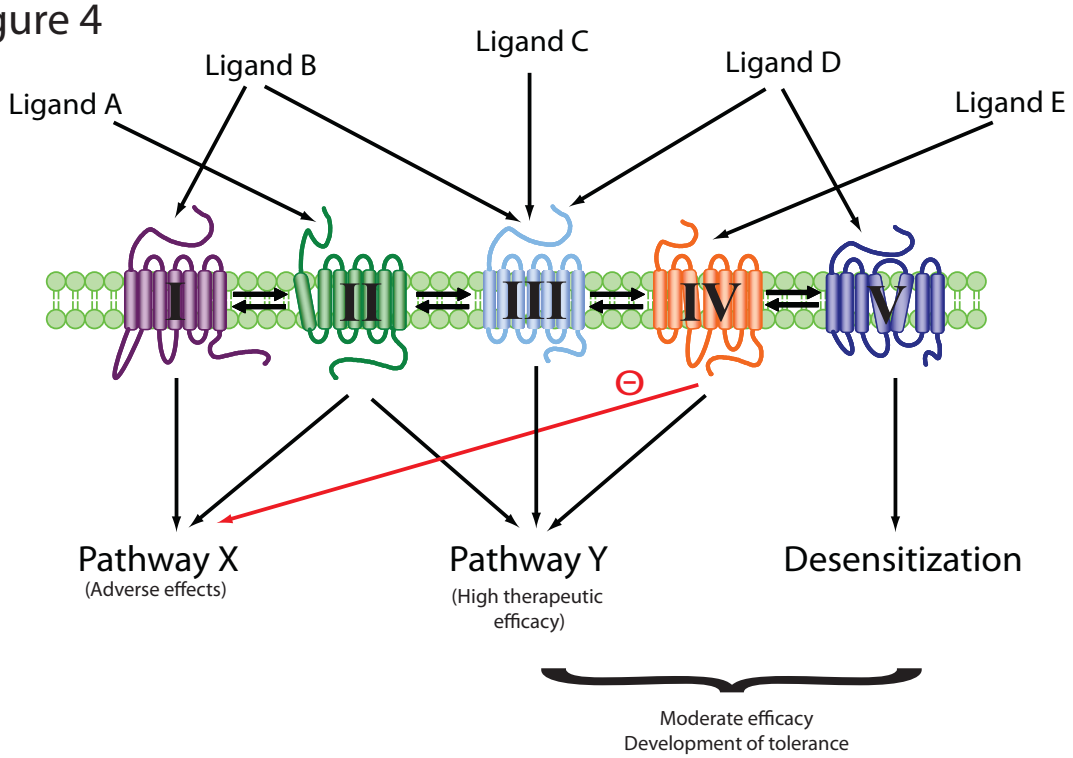

Figure 4 – Exploiting functional selectivity in drug discovery. In the simplified example shown here, we present strategies for the design of compounds for a receptor that couples to two independent signaling pathways: Pathway X, which leads to adverse effects, and Pathway Y, which is responsible for the desired therapeutic response. In this scenario, both **Ligand A** and **Ligand B** would provide therapeutic benefit but would also elicit unwanted side effects. The mechanism underlying this profile differs among these ligands. Ligand A does so by stabilizing a receptor conformation (II) that can couple to both pathways. Ligand B, on the other hand, exhibits the same clinical profile by stabilizing two distinct receptor conformations (I and III) that respectively couple to each pathway selectively. **Ligand C** would represent a suitable therapeutic strategy by selectively coupling to Pathway Y to provide clinical benefit without the side effects produced by Pathway X. **Ligand D**, despite selectively coupling to Pathway Y, may not be as clinically-effective as Ligand C due to its ability to stabilize a receptor conformation (V) that can become desensitized to signaling, a response that often leads to the development of tolerance to the drug. However, by selectively activating Pathway Y and eliciting inverse agonism towards Pathway X, **Ligand E** could prove to exhibit the greatest clinical response of them all.

References:

1. Lappano, R. & Maggiolini, M. G protein-coupled receptors: novel targets for drug discovery in cancer. *Nature Reviews Drug Discovery* **10**, 47–60(2011).

2. Pierce, K.L., Premont, R.T. & Lefkowitz, R.J. Seven-transmembrane receptors. *Nature reviews. Molecular cell biology* **3**, 639-50(2002).

3. Stephenson, R.P. A modification of receptor theory. *British journal of pharmacology* **11**, 379-393(1956).

4. Chidiac, P., Hebert T.E., Valiquette, M., et al. Inverse agonist activity of beta-adrenergic antagonists. *Molecular pharmacology* **45**, 490-9(1994).

5. Kenakin, T. Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Molecular pharmacology* **65**, 2-11(2004).

6. Bond, R., Leff, P., Johnson, T., et al. Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta2-adrenoceptor. *Nature* **374**, 272-276(1995).

7. Costa, T. & Herz, A. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7321-5(1989).

8. Samama, P., Cotecchia, S., Costa, T., et al. A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *The Journal of biological chemistry* **268**, 4625-36(1993).

9. Samama, P., Pei, G., Costa, T., et al. Negative Antagonists Promote an Inactive Conformation of the beta2-Adrenergic Receptor. *Molecular pharmacology* **45**, 390-394(1994).

10. Lefkowitz, R.J., Cotecchia, S., Samama, P., et al. Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends in pharmacological sciences* **14**, 303-7(1993).

11. Hermans, E. Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacology & Therapeutics* **99**, 25-44(2003).

12. Bockaert, J., Fagni, L., Dumuis, A., et al. GPCR interacting proteins (GIP). *Pharmacology & therapeutics* **103**, 203-21(2004).

13. Brady, A. & Limbard, L. G protein-coupled receptor interacting proteins: Emerging roles in localization and signal transduction. *Cellular Signalling* **14**, 297-309(2002).

14. Urban, J.D., Clarke, W., von Zastrow, M., et al. Functional Selectivity and Classical Concepts of Quantitative Pharmacology. *Pharmacology* **320**, 1-13(2007).

15. Kenakin, T. Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends in pharmacological sciences* **16**, 232-8(1995).

16. Spengler, D., Waeber, C., Pantaloni, C., et al. Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365**, 170-175(1993).

17. Robb, S., Cheek, T.R., Hannan, F.L., et al. Agonist-specific coupling of a cloned Drosophila octopamine/tyramine receptor to multiple second messenger systems. *The EMBO Journal* **13**, 1325(1994).

18. Mottola, D.M., Kilts, J.D., Lewis, M.M., et al. Functional selectivity of dopamine receptor agonists. I. Selective activation of postsynaptic dopamine D2 receptors linked to adenylate cyclase. *The Journal of pharmacology and experimental therapeutics* **301**, 1166-78(2002).

19. Kilts, J.D., Connery, H.S., Arrington, E.G., et al. Functional selectivity of dopamine receptor agonists. II. Actions of dihydrexidine in D2L receptor-transfected MN9D cells and pituitary lactotrophs. *The Journal of pharmacology and experimental therapeutics* **301**, 1179-89(2002).

20. Sato, M., Horinouchi, T., Hutchinson, D.S., et al. Ligand-directed signaling at the beta3-adrenoceptor produced by 3-(2-Ethylphenoxy)-1-[(1,S)-1,2,3,4-tetrahydronapth-1-ylamino]-2S-2-propanol oxalate (SR59230A) relative to receptor agonists. *Molecular pharmacology* **72**, 1359-68(2007).

21. De Deurwaerdère, P., Navailles, S., Berg, K.A., et al. Constitutive activity of the serotonin2C receptor inhibits in vivo dopamine release in the rat striatum and nucleus accumbens. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **24**, 3235-41(2004).

22. Berg, K., Maayani, S., Goldfarb, J., et al. Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Molecular pharmacology* **54**, 94-104(1998).

23. Reiner, S., Ambrosio, M., Hoffmann, C., et al. Differential signaling of the endogenous agonists at the {beta}2-adrenergic receptor. *The Journal of biological chemistry* **285**, 36188 -36198(2010).

24. Kohout, T., Nicholas, S.L., Perry, S.J., et al. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *The Journal of biological chemistry* **279**, 23214-22(2004).

25. Nickolls, S.A., Fleck, B., Hoare, S.R.J., et al. Functional Selectivity of Melanocortin 4 Receptor Peptide and Nonpeptide Agonists : Evidence for Ligand-Specific Conformational States. *Pharmacology* **313**, 1281-1288(2005).

26. Rajagopal, S., Rajagopal, K. & Lefkowitz, R.J. Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nature reviews. Drug discovery* **9**, 373-86(2010).

27. Luttrell, L.M. & Gesty-Palmer, D. Beyond Desensitization : Physiological Relevance of Arrestin-Dependent Signaling. *Pharmacological Reviews* **62**, 305-330(2010).

28. Violin, J.D., Dewire, S.M., Yamashita, D., et al. Selectively Engaging beta-Arrestins at the Angiotensin II Type 1 Receptor Reduces Blood Pressure and Increases Cardiac. *Journal of Pharmacology and Experimental Therapeutics* **335**, 572-579(2010).

29. Azzi, M., Charest, P.G., Angers, S., et al. Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11406-11(2003).

30. Werry, T.D., Gregory, K.J., Sexton, P.M., et al. Characterization of serotonin 5-HT2C receptor signaling to extracellular signal-regulated kinases 1 and 2. *Journal of neurochemistry* **93**, 1603-15(2005).

31. Audet, N., Paquin-Gobeil, M., Landry-Paquet, O., et al. Internalization and Src activity regulate the time course of ERK activation by delta opioid receptor ligands. *The Journal of biological chemistry* **280**, 7808-16(2005).

32. Galandrin, S. & Bouvier, M. Distinct signaling profiles of beta1 and beta2 adrenergic receptor ligands toward adenylyl cyclase and mitogen-activated protein kinase reveals the pluridimensionality of efficacy. *Mol Pharmacol* **70**, 1575-1584(2006).

33. Wisler, J.W., Dewire, S.M., Whalen, E.J., et al. A unique mechanism of beta-blocker action Carvedilol stimulates beta-arrestin signaling. *Proceedings of the National Academy of Sciences* **104**, 16657-16662(2007).

34. Metra, M., Cas, L.D., di Lenarda, A., et al. Beta-blockers in heart failure: are pharmacological differences clinically important? *Heart failure reviews* **9**, 123-30(2004).

35. Gainetdinov, R.R., Premont, R.T., Bohn, L.M., et al. Desensitization of G protein-coupled receptors and neuronal functions. *Annual review of neuroscience* **27**, 107-44(2004).

36. Wolfe, B.L. & Trejo, J. Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. *Traffic (Copenhagen, Denmark)* **8**, 462-70(2007).

37. Kelly, E., Bailey, C.P. & Henderson, G. Agonist-selective mechanisms of GPCR desensitization. *British journal of pharmacology* **153 Suppl** , S379-88(2008).

38. Trester-Zedlitz, M., Burlingame, A., Kobilka, B., et al. Mass spectrometric analysis of agonist effects on posttranslational modifications of the beta-2 adrenoceptor in mammalian cells. *Biochemistry* **44**, 6133-43(2005).

39. Luk, T., Jin, W., Zvonok, A., et al. Identification of a potent and highly efficacious, yet slowly desensitizing CB1 cannabinoid receptor agonist. *British journal of pharmacology* **142**, 495-500(2004).

40. Stout, B.D., Clarke, W.P. & Berg, K. Rapid desensitization of the serotonin(2C) receptor system: effector pathway and agonist dependence. *The Journal of pharmacology and experimental therapeutics* **302**, 957-62(2002).

41. Roettger, B., Ghanekar, D., Rao, R., et al. Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Molecular pharmacology* **51**, 357(1997).

42. Pheng, L.H., Dumont, Y., Fournier, A., et al. Agonist- and antagonist-induced sequestration/internalization of neuropeptide Y Y1 receptors in HEK293 cells. *British journal of pharmacology* **139**, 695-704(2003).

43. Sneddon, W.B., Syme, C.A., Bisello, A., et al. Activation-independent parathyroid hormone receptor internalization is regulated by NHERF1 (EBP50). *The Journal of biological chemistry* **278**, 43787-96(2003).

44. Bhowmick, N., Narayan, P. & Puett, D. The endothelin subtype A receptor undergoes agonist- and antagonist-mediated internalization in the absence of signaling. *Endocrinology* **139**, 3185-92(1998).

45. Gray, J.A. & Roth, B.L. Paradoxical trafficking and regulation of 5-HT(2A) receptors by agonists and antagonists. *Brain research bulletin* **56**, 441-51(2001).

46. Pradhan, A.A.A., Walwyn, W., Nozaki, C., et al. Ligand-Directed Trafficking of the delta-Opioid Receptor In Vivo: Two Paths Toward Analgesic Tolerance. *Journal of Neuroscience* **30**, 16459-16468(2010).

47. Kenakin, T. New concepts in drug discovery: collateral efficacy and permissive antagonism. *Nature reviews. Drug discovery* **4**, 919-27(2005).

48. Thomas, W.G., Qian, H., Chang, C.S., et al. Agonist-induced phosphorylation of the angiotensin II (AT(1A)) receptor requires generation of a conformation that is distinct from the inositol phosphate-signaling state. *The Journal of biological chemistry* **275**, 2893-900(2000).

49. Zidar, D., Violin, J.D., Whalen, E.J., et al. Selective engagement of G protein coupled receptor kinases (GRKs) encodes distinct functions of biased ligands. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 9649-54(2009).

50. Johnson, E.A., Oldfield, S., Braksator, E., et al. Agonist-Selective Mechanisms of mu-Opioid Receptor Desensitization in Human Embryonic Kidney 293 Cells. *Molecular pharmacology* **70**, 676(2006).

51. Whistler, J.L. & Zastrow, M. von Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9914-9919(1998).

52. Chu, J., Zheng, H., Loh, H.H., et al. Morphine-induced mu-opioid receptor rapid desensitization is independent of receptor phosphorylation and beta-arrestins. *Cellular signalling* **20**, 1616-24(2008).

53. Zeitz, K.P., Malmberg, A.B., Gilbert, H., et al. Reduced development of tolerance to the analgesic effects of morphine and clonidine in PKC gamma mutant mice. *Pain* **94**, 245-53(2001).

54. Smith, F.L., Gabra, B.H., Smith, P.A., et al. Determination of the role of conventional, novel and atypical PKC isoforms in the expression of morphine tolerance in mice. *Pain* **127**, 129-39(2007).

55. Terman, G.W., Jin, W., Cheong, Y.-P., et al. G-protein receptor kinase 3 (GRK3) influences opioid analgesic tolerance but not opioid withdrawal. *British journal of pharmacology* **141**, 55-64(2004).

56. Ferguson, S.S., Zhang, J., Barak, L.S., et al. Molecular mechanisms of G protein-coupled receptor desensitization and resensitization. *Life sciences* **62**, 1561-5(1998).

57. Finn, A.K. & Whistler, J.L. Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* **32**, 829-39(2001).

58. Berger, A.C. & Whistler, J.L. How to design an opioid drug that causes reduced tolerance and dependence. *Annals of neurology* **67**, 559–569(2010).

59. Vaidehi, N. & Kenakin, T. The role of conformational ensembles of seven transmembrane receptors in functional selectivity. *Current opinion in pharmacology* **10**, 775-781(2010).

60. Kobilka, B.K. & Deupi, X. Conformational complexity of G-protein-coupled receptors. *Trends in pharmacological sciences* **28**, 397-406(2007).

61. Gether, U., Lin, S. & Kobilka, B.K. Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes. *The Journal of biological chemistry* **270**, 28268-75(1995).

62. Swaminath, G., Xiang, Y., Lee, T.W., et al. Sequential binding of agonists to the beta2 adrenoceptor. Kinetic evidence for intermediate conformational states. *The Journal of biological chemistry* **279**, 686-91(2004).

63. Ghanouni, P., Gryczynski, Z., Steenhuis J.J., et al. Functionally different agonists induce distinct conformations in the G protein coupling domain of the beta 2 adrenergic receptor. *The Journal of biological chemistry* **276**, 24433-6(2001).

64. Yao, X., Parnot, C., Deupi, X., et al. Coupling ligand structure to specific conformational switches in the beta2-adrenoceptor. *Nature chemical biology* **2**, 417-22(2006).

65. Vilardaga, J.-P., Steinmeyer, R., Harms, G.S., et al. Molecular basis of inverse agonism in a G protein-coupled receptor. *Nature chemical biology* **1**, 25-8(2005).

66. Bisello, A., Chorev, M., Rosenblatt, M., et al. Selective ligand-induced stabilization of active and desensitized parathyroid hormone type 1 receptor conformations. *The Journal of biological chemistry* **277**, 38524-30(2002).

67. Li, J.H., Han, S.-J., Hamdan, F.F., et al. Distinct structural changes in a G protein-coupled receptor caused by different classes of agonist ligands. *The Journal of biological chemistry* **282**, 26284-93(2007).

68. Li, J.H., Hamdam, F.F., Kim, S.-K., et al. Ligand-specific changes in M3 muscarinic acetylcholine receptor structure detected by a disulfide scanning strategy. *Biochemistry* **47**, 2776-88(2008).

69. Audet, N., Gales, C., Archer-Lahlou, E., et al. Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing delta-opioid receptors and heterotrimeric G proteins. *The Journal of biological chemistry* **283**, 15078-88(2008).

70. Alves, I.D., Salamon, Z., Varga, E., et al. Direct observation of G-protein binding to the human delta-opioid receptor using plasmon-waveguide resonance spectroscopy. *The Journal of biological chemistry* **278**, 48890-7(2003).

71. Yao, X.J., Velez Ruiz, G., Whorton, M.R., et al. The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 9501-6(2009).

72. Galés, C., Van Durm, J.J.J., Schakk, S., et al. Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nature structural & molecular biology* **13**, 778-86(2006).

73. Nikolaev, V.O., Hoffmann, C., Bunemann, M., et al. Molecular basis of partial agonism at the neurotransmitter alpha2A-adrenergic receptor and Gi-protein heterotrimer. *The Journal of biological chemistry* **281**, 24506-11(2006).

74. Wei, H., Ahn, S., Shenoy, S.K., et al. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10782-7(2003).

75. Galandrin, S., Oligny-Longpre, G., Bonin, H., et al. Conformational rearrangements and signaling cascades involved in ligand-biased mitogen-activated protein kinase signaling through the beta1-adrenergic receptor. *Molecular pharmacology* **74**, 162-72(2008).

76. Rosenbaum, D.M., Rasmussen, S.G.F. & Kobilka, B.K. The structure and function of G-protein-coupled receptors. *Nature* **459**, (2009).

77. Cherezov, V., Rosenbaum, D.M., Hanson, M., et al. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science (New York, N.Y.)* **318**, 1258-65(2007).

78. Chien, E.Y.T., Liu, W., Zhao, Q., et al. Structure of the Human Dopamine D3 Receptor in Complex with a D2/D3 Selective Antagonist. *Science* **330**, 1091-1095(2010).

79. Rasmussen, S.G.F., Choi, H.-J., et al. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* **450**, 383-7(2007).

80. Warne, T., Serrano-Vega, M.J., et al. Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**, 486-91(2008).

81. Jaakola, V.P., Griffith, M.T., Hanson, M.A., et al. The 2.6 Angstrom Crystal Structure of a Human A2A Adenosine Receptor Bound to an Antagonist. *Science* **322**, 1211(2008).

82. Wu, B., Chien, E.Y.T., Mol, C.D., et al. Structures of the CXCR4 Chemokine GPCR with Small-Molecule and Cyclic Peptide Antagonists. *Science* **1066**, (2010).

83. Rasmussen, S.G.F., Choi, H.-J., Fung, J.J., et al. Structure of a nanobody-stabilized active state of the β2 adrenoceptor. *Nature* **469**, 175-180(2011).

84. Rosenbaum, D.M., Zhang, C., Lyons, J., et al. Structure and function of an irreversible agonist-β2 adrenoceptor complex. *Nature* **469**, 236-240(2011).

85. Warne, T., Moukhametzianov, R., Baker, J.G., et al. The structural basis for agonist and partial agonist action on a β1-adrenergic receptor. *Nature* **469**, 241-244(2011).

86. Goncalves, J., South, K., Ahuja, S., et al. Highly conserved tyrosine stabilizes the active state of rhodopsin. *Proceedings of the National Academy of Sciences of the United States of America* **107**, (2010).

87. Bokoch, M.P., Zou, Y., Rasmussen, S.G.F., et al. Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature* **463**, 108-12(2010).

88. Stewart, G.D., Sexton, P.M. & Christopoulos, A. Prediction of functionally selective allosteric interactions at an M3 muscarinic acetylcholine receptor mutant using Saccharomyces cerevisiae. *Molecular pharmacology* **78**, 205-14(2010).

89. Stewart, G.D., Sexton, P.M. & Christopoulos, A. Detection of novel functional selectivity at M3 muscarinic acetylcholine receptors using a Saccharomyces cerevisiae platform. *ACS chemical biology* **5**, 365-75(2010).

90. Stewart, G.D., Valant, C., Dowell, S.J., et al. Determination of adenosine A1 receptor agonist and antagonist pharmacology using Saccharomyces cerevisiae: implications for ligand screening and functional selectivity. *The Journal of pharmacology and experimental therapeutics* **331**, 277-86(2009).

91. Jiang, L.I., Collins, J., Davis, R., et al. Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *The Journal of biological chemistry* **282**, 10576-84(2007).

92. Charest, P.G., Terrillon, S. & Bouvier, M. Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET. *EMBO reports* **6**, 334-40(2005).

93. Breton, B., Lagacé, M. & Bouvier, M. Combining resonance energy transfer methods reveals a complex between the {alpha}2A-adrenergic receptor, G{alpha}i1{beta}1{gamma}2, and GRK2. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 1-11(2010).doi:10.1096/fj.10-164061

94. Gorokhovatsky, A.Y., Marchenkov, V.V., Rudenko, N.V., et al. Fusion of Aequorea victoria GFP and aequorin provides their Ca(2+)-induced interaction that results in red shift of GFP absorption and efficient bioluminescence energy transfer. *Biochemical and biophysical research communications* **320**, 703-11(2004).

95. Huwiler, K.G., Machleidt, T., Chase, L., et al. Characterization of serotonin 5-hydroxytryptamine-1A receptor activation using a phospho-extracellular-signal regulated kinase 2 sensor. *Analytical biochemistry* **393**, 95-104(2009).

96. Fosbrink, M., Aye-Han, N.-N., Cheong, R., et al. Visualization of JNK activity dynamics with a genetically encoded fluorescent biosensor. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5459-64(2010).

97. Hodgson, L., Shen, F. & Hahn, K. Biosensors for characterizing the dynamics of rho family GTPases in living cells. *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]* **Chapter 14**, Unit 14.11.1-26(2010).

98. Depry, C. & Zhang, J. Visualization of kinase activity with FRET-based activity biosensors. *Current protocols in molecular biology / edited by Frederick M. Ausubel ... [et al.]* **Chapter 18**, Unit 18.15(2010).

99. Nishioka, T., Frohman, M.A., Matsuda, M., et al. Heterogeneity of Phosphatidic Acid Levels and Distribution at the Plasma Membrane in Living Cells as Visualized by a Forster Resonance Energy Transfer (FRET) Biosensor. *The Journal of biological chemistry* **285**, 35979-87(2010).

100. Depry, C., Allen, M.D. & Zhang, J. Visualization of PKA activity in plasma membrane microdomains. *Molecular bioSystems* **7**, 52-8(2010).

101. Lin, C., Kolossov, V.L., Tsvid, G., et al. Imaging in real-time with FRET the redox response of tumorigenic cells to glutathione perturbations in a microscale flow. *Integrative biology : quantitative biosciences from nano to macro* (2010).doi:10.1039/c0ib00071j

102. Harvey, C.D., Ehrhardt, A.G., Cellurale, C., et al. A genetically encoded fluorescent sensor of ERK activity. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 19264-9(2008).

103. Breton, B., Sauvageau, E., Zhou, J., et al. Multiplexing of multicolor bioluminescence resonance energy transfer. *Biophysical journal* **99**, 4037-46(2010).

104. Scott, C.W. & Peters, M.F. Label-Free Whole-Cell Assays: Expanding the Scope of GPCR Screening. *Drug discovery today* **15**, 704-716(2010).

105. Yu, N., Atienza, J.M., Bernard, J., et al. Real-time monitoring of morphological changes in living cells by electronic cell sensor arrays: an approach to study G protein-coupled receptors. *Analytical chemistry* **78**, 35-43(2006).

106. Fang, Y., Li, G. & Ferrie, A.M. Non-invasive optical biosensor for assaying endogenous G protein-coupled receptors in adherent cells. *Journal of pharmacological and toxicological methods* **55**, 314-22

107. Schröder, R., Janssen, N., Schmidt, J. et al. Deconvolution of complex G protein-coupled receptor signaling in live cells using dynamic mass redistribution measurements. *Nature biotechnology* **28**, 943-949(2010).

108. Peters, M.F. & Scott, C.W. Evaluating Cellular Impedance Assays for Detection of GPCR Pleiotropic Signaling and Functional Selectivity. *Journal of Biomolecular Screening* **14**, 246-255(2009).

109. Dodgson, K., Gedge, L., Murray, D.C., et al. A 100K well screen for a muscarinic receptor using the Epic label-free system--a reflection on the benefits of the label-free approach to screening seven-transmembrane receptors. *Journal of receptor and signal transduction research* **29**, 163-72(2009).

110. McGuinness, R.P., Proctor, J.M., Gallant, D.L., et al. Enhanced selectivity screening of GPCR ligands using a label-free cell based assay technology. *Combinatorial chemistry & high throughput screening* **12**, 812-23(2009).

111. Gregory, K.J., Hall, N.E., Tobin A.B., et al. Identification of orthosteric and allosteric site mutations in M2 muscarinic acetylcholine receptors that contribute to ligand-selective signaling bias. *The Journal of biological chemistry* **285**, 7459-74(2010).

112. Kenakin, T. & Miller, L.J. Seven Transmembrane Receptors as Shapeshifting Proteins : The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. *Pharmacological Reviews* **62**, 265-304(2010).

113. Valant, C., Aurelio, L., Urmaliya, V.B., et al. Delineating the mode of action of adenosine A1 receptor allosteric modulators. *Molecular pharmacology* **78**, 444-55(2010).

114. Koole, C., Wootten, D., Simms, J., et al. Allosteric ligands of the glucagon-like peptide 1 receptor (GLP-1R) differentially modulate endogenous and exogenous peptide responses in a pathway-selective manner: implications for drug screening. *Molecular pharmacology* **78**, 456-65(2010).

115. Evans, B.A., Broxton, N., Merlin, J., et al. Quantification of Functional Selectivity at the Human {alpha}1A-Adrenoceptor. *Molecular pharmacology* **79**, 298-307(2011).

116. Figueroa, K.W., Griffin, M.T. & Ehlert, F.J. Selectivity of agonists for the active state of M1 to M4 muscarinic receptor subtypes. *The Journal of pharmacology and experimental therapeutics* **328**, 331-42(2009).

117. Christopoulos, A. Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends in pharmacological sciences* **19**, 351-7(1998).